ORAL TOLERANCE TO SOLUBLE PROTEIN ANTIGENS IN HUMANS

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SUMMARY

Oral tolerance describes the down-regulation of the systemic immune response to antigens that are encountered by the oral route. It is a well described phenomenon in many animal studies, but there is little work on oral tolerance in humans. Animal studies suggest that there are several different immune mechanisms that mediate oral tolerance, including clonal deviation, active suppression, clonal anergy and clonal deletion. There are several factors that influence which mechanism induces the production of tolerance including the type of antigen, the animal model used and most critically the length and dose of feeding. Oral tolerance has been shown to occur in humans, but there are very few published studies investigating the phenomenon.

This thesis describes my work in developing a protocol to investigate oral tolerance to soluble protein antigens in humans. I investigated oral tolerance to two protein antigens – namely keyhole limpet haemocyanin (KLH) and ovalbumin (OVA). KLH is a neoantigen that is not normally encountered by humans, and therefore allowed me to investigate oral tolerance to low dose feeding. A control group was immunised with KLH and the immune response was assessed by testing humoral responses, delayed type hypersensitivity (DTH) responses and *in vitro* lymphocyte proliferation. To assess oral tolerance, a study group was fed a course of KLH prior to receiving the same immunisation schedule. Three feeding schedules were used. The immune response was measured in the same way as the control group and any differences between the two were attributed to oral tolerance.

Immunisation with KLH caused a good immune response with positive DTH responses, the production of anti-KLH IgG and IgA and detectable *in vitro* lymphocyte proliferation. The immune response in the group fed the lowest dose of KLH (10mg of KLH fed for 10 consecutive days) showed no differences from the control group. After feeding the intermediate dose of KLH (50mg of KLH fed for 10 consecutive days), there was detectable *in vitro* lymphocyte proliferation, but no humoral response. After immunisation the DTH

responses were significantly reduced, the *in vitro* lymphocyte proliferation responses were unchanged and anti-KLH IgG, but not IgA, production was increased compared to the control group. These changes demonstrate that oral tolerance in the T cell compartment does occur in humans. The group fed the largest dose of KLH (50mg of KLH fed for 15 days in two separate blocks) had detectable *in vitro* lymphocyte proliferation after feeding. After immunisation, there were reduced DTH to KLH, detectable anti-KLH IgG and IgA (but not significantly different from the control group) and detectable *in vitro* lymphocyte proliferation. Compared to the group fed the intermediate dose of KLH, the *in vitro* lymphocyte proliferation was significantly greater after feeding, but significantly less after immunisation. Thus oral tolerance in the T cell compartment was also demonstrated in this group.

The mechanisms responsible for the induction of tolerance are unknown. The results above are consistent with the induction of an immunoregulatory cell, although they would also be consistent with the induction of other mechanisms of tolerance. Efforts were made to identify this putative immunoregulatory cell by assaying the cytokine profile produced from cell cultures, by measuring the levels of IgG subclasses induced and by using flow cytometry to identify cell surface markers of cells cultured with KLH in both the control group and the KLH-fed groups. The data obtained could not identify any immunoregulatory cell.

OVA is a common dietary protein and was used to investigate oral tolerance to prolonged courses of feeding. Volunteers were immunised with OVA using the same schedule as that used for KLH and the same assays were used to investigate the immune response. Two doses of immunisation were used. At baseline (i.e. after feeding) there were low levels of anti-OVA IgA and IgG but no detectable *in vitro* lymphocyte proliferation. The lower dose immunisation schedule did not cause any detectable immune response. The higher dose immunisation schedule did not induce any detectable *in vitro* lymphocyte proliferation response or DTH response, but did cause an increase in either anti-OVA IgG or IgA in three of the four volunteers investigated. These results suggest that oral tolerance to OVA is more

pronounced than to KLH. One can hypothesise that different mechanisms of tolerance such as clonal anergy have been induced. Attempts to provide supportive evidence for this hypothesis by demonstrating reversal of anergy by pre-culture of cells with IL-2 failed.

CONTENTS

TABLES AND FIGURES

ABBREVIATIONS

APS	ammonium persulfate
BSA	bovine serum albumin
CD	Crohn's disease
cDNA	complimentary deoxyribonucleic acid
conA	concanavalin A
DDW	doubly distilled water
DEA	diethanolamine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNP	dinitrophenylate
DTH	delayed type hypersensitivity
EAE	experimental aytoimmuneencephalitis
EAU	experimental autoimmune uveoretinitis
ELISA	enzyme linked immunosorbent assay
FITC	fluorescein isothiocyanate
g	gravity
KLH	keyhole limpet haemocyanin
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
M	molar
MAdCAM-1	mucosal addressin cellular adhesion molecule-1
mg	milligram
ml	millilitre
mM	millimolar
MMLV	maloney leukaemia virus
µg	microgram
µl	microlitre
mRNA	messenger ribosomal nucleic acid
ng	nanogram
nm	nanometer

NOD	non-obese diabetic
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PE	phycoerythrin
PNPP	p-nitrophenylphosphate
PPD	purified protein derivative
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
TNF	tumour necrosis factor
tTG	tissue transglutaminase
Vol	volume
WHO	World Health Organisation
Wt	weight

PRESENTATIONS

The following presentations have been based on work contained within this thesis:

1) Lymphocyte proliferation responses to oral soluble antigens – length and dose of feeding of soluble antigen influences immune outcome, J.R. Boulton-Jones, A. Ferguson – oral presentation to 6th UEGW, October 1997. Published as an abstract in Gut Suppl.3, Vol.41, Page A71.

2) Oral tolerance in humans – duration of feeding alters the mechanisms of tolerance production to soluble protein antigens, J.R. Boulton-Jones, A. Ferguson – oral presentation to the BSG, March 1998 and oral presentation to the AGA, May 1998. Published as an abstract in Gut Suppl.1, Vol.42, Page A23.

3) Oral tolerance in humans – evidence for active suppression as a mechanism of tolerance production to soluble protein antigens? J.R. Boulton-Jones, A. Ferguson – poster presentation to the BSG, March 1998 and poster presentation to the AGA, May 1998. Published as an abstract in Gut Suppl.1, Vol.42, Page A85.

4) Pre-feeding soluble protein antigen causes reduced cytokine mRNA expression in antigen-specific peripheral blood lymphocytes in healthy human volunteers, J.R. Boulton-Jones, A. Ferguson – poster presentation to the BSG, March 1999. Published as an abstract in Gut Suppl.1, Vol.44, Page A71.

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Lastly I would like to thank my family for their support and encouragement during the writing of the thesis.

DECLARATION

I declare that this thesis has been composed by me and that the work contained within it was performed by me, except where stated otherwise. The entire work was performed while I was employed as a research lecturer by the University of Edinburgh working at the Gastrointestinal Laboratory, Department of Medicine, Western General Hospital, Edinburgh. The thesis has not been submitted for any other professional qualification.

1 AIMS OF THIS THESIS

One of the mysteries of the immune system is that ingested food antigens do not produce an immunological reaction in the small intestinal mucosa. In some way, we develop tolerance to the numerous antigens presented to us in our intestines but the mechanisms by which this is accomplished, particularly in humans, are obscure. In some way, orally encountered antigen causes down-regulation of the systemic immune response to that antigen. Oral tolerance is likely to have evolved as a protective mechanism to prevent the development of wasteful, and possibly harmful, immune reactions to harmless, ingested antigens (such as those in food proteins) in the gut mucosa. It is a very robust phenomenon that occurs with many different antigens and in many different animal species. The mechanisms leading to oral tolerance are being elucidated in animal models. It appears that several different mechanisms of tolerance production occur in different models and even within the same model. Factors that influence the type of tolerance produced include the animal model system used, age, species and the type and dose of antigen given.

Oral tolerance may be important in clinical practice in two ways. Firstly, diseases of gastrointestinal inflammation such as coeliac disease and inflammatory bowel disease (IBD) may result from a breakdown of tolerance. An improved understanding of oral tolerance may therefore offer new insight into the pathogenesis of these diseases, and potentially offer new immunomodulatory opportunities for their treatment. Secondly, feeding antigen offers a cheap, safe way to suppress immune responses in an antigen specific manner. Investigators have therefore suggested that oral tolerance could be used to treat many diseases of abnormal inflammation and particularly autoimmune diseases. This method of treatment has been successfully applied to animal models of autoimmune disease, but has not yet proved successful in humans. These two areas of clinical applications have resulted in an interest in oral tolerance, and led to a plethora of animal studies into the phenomenon.

Oral tolerance has been demonstrated in humans. However, I am aware of only one study investigating oral tolerance in humans in a controlled manner, and no published studies looking at the mechanisms of induction of oral tolerance in humans. The aims of this thesis therefore are as follows:

1) To develop a model to confirm that tolerance does indeed occur in humans.
2) To assess the effect of oral feeding on different aspects of the systemic immune response, including cellular and humoral immune responses.
3) To assess the effect of different doses and types of antigen on the development of oral tolerance.
4) To develop theories of the mechanisms of tolerance production by comparing the type of immune response generated to those found in animal model of tolerance.
5) To develop experimental protocols to test one or more of these theories.

In this thesis, I will review the literature on oral tolerance, concentrating particularly on the animal studies examining the mechanisms of induction of tolerance. I will then detail my experience of developing a protocol to investigate oral tolerance in humans. I will also present my experience of the effects of altering the type and dose of antigen on the immune responses of oral tolerance. The possible mechanisms of tolerance induction in humans will be discussed and experimental protocols devised to attempt to investigate which of these mechanisms is relevant to humans will be presented.

2 INTRODUCTION AND LITERATURE REVIEW

2.1 A Brief History of Oral Tolerance

2.1.1 A Definition of Oral Tolerance

Oral tolerance can be defined as the down-regulation of the systemic immune response to a specific antigen when that antigen is encountered by the oral route.

2.1.2 An Introduction to Tolerance

The immune system has evolved to detect and destroy harmful invasive organisms such as viruses, bacteria and parasites. A powerful array of defensive mechanisms has evolved to neutralise and kill such organisms. To prevent these destructive mechanisms being induced to non-pathogenic antigens, the immune system has to be able to recognise these antigens as harmless and avoid mounting a potentially damaging response to them. The mechanisms of tolerance have developed to allow this to happen. The most important form of tolerance is self-tolerance. The body can recognise self-antigens and avoid mounting an immune response to them. If these mechanisms fail, the result is auto-immune disease, with all the deleterious health effects that follow.

Other antigens apart from self antigens are also harmless, and immune responses to them are wasteful or damaging. One such group of antigens is that encountered at mucosal sites including dietary antigens that are absorbed through mucosa of the gastrointestinal tract. These antigens are harmless and indeed an immune response against them can be positively dangerous to the host, as is demonstrated by the gastrointestinal damage that is caused by the immune response to gliadin in coeliac disease. This thesis looks at the mechanisms of oral tolerance, which have evolved to prevent immune responses to orally encountered antigens.

2.1.3 The Early History of Oral Tolerance

There are anecdotal reports dating back to the last century when South American Indians were said to eat the leaves of poison ivy plants to prevent the development of contact sensitivity reactions later[1]. Although at the time these reports were treated with scepticism or assumed to be due to the superstitions of a primitive people, a rational, scientific explanation for this behaviour came with the early studies of oral tolerance. The first scientific report describing oral tolerance was published at the start of the century when it was found that guinea pigs prefed ovalbumin (OVA) did not develop anaphylaxis when inoculated with it systemically, whereas non-fed controls did[2;3]. In 1946, Chase tried a variety of ways to reduce the severity of contact hypersensitivity and was surprised to find feeding animals (guinea pigs in this case) the sensitisor prior to cutaneous exposure reduced the severity of the subsequent response[4]. He went on to demonstrate the antigen specificity of the response by showing that prefed animals still developed contact hypersensitivity to unrelated chemicals. This antigen specificity of oral tolerance is an important feature of the phenomenon that has been consistently demonstrated over the years.

2.1.4 The Range of Oral Tolerance

Subsequent investigators have confirmed the concept of oral tolerance and expanded it in a number of important ways. Firstly it has been shown to occur in many different species, including mice[5;6], dogs[7], pigs[8] and humans[9]. Secondly, it was found that humoral as well as cell mediated immunity (CMI) could be affected[10]. Indeed, both systemic and secretory humoral immunity could be suppressed[11]. Indirect evidence that oral tolerance of secretory immunity can be induced is provided by the observation that secretory immunoglobulin (Ig) A antibodies to food antigens are rare in humans[12]. A wide variety of antigens were shown to produce a similar response, including contact sensitisers[4;5;13], soluble proteins[9;10] and particulate antigens such as heterologous red blood cells[11;14], or bacteria[6]. Lastly, it was shown that tolerance could be induced in animals that had already had a systemic immune

response stimulated by parental injection of the same antigen[15;16]. These findings confirmed oral tolerance to be a widespread and reproducible immune response.

2.2 The Immune Features of Oral Tolerance

There are, however, a number of differences between oral tolerance induced in the humoral and cell mediated limbs of the immune system. CMI appears to be the easier to suppress, and as little as one dose of antigen administered orally is required in some experimental models[6;17]. The response of the systemic humoral system to oral antigen is more complex. In certain experimental models, the level of circulating antibody rises in response to oral antigen[10;18]. Priming occurs in other cases - i.e. although there is no difference in antibody levels after feeding, the antibody response is of greater magnitude than it would otherwise have been following subsequent parenteral exposure[9]. In other cases, the systemic antibody response is reduced after subsequent parenteral exposure[15;19]. Different classes of Ig also differ in their susceptibility to tolerisation. IgE on the whole is easy to suppress, with IgG having an intermediate position and IgA being the hardest to suppress and in fact levels can often be primed[15;20]. Secretory antibody generally is increased or primed after oral exposure[6;9]. It is possible in some transfer experiments, however, to demonstrate suppression even of Peyer's patch Ig responses[11]. Indirect evidence for the induction of tolerance of secretory IgA comes from the observation that it is difficult to detect secretory IgA to normal food antigens[12]. Another general principle appears to be that the easier it is to induce tolerance, the more long-lasting it is. For example, CMI can be suppressed for up to 18 months by a single feed in mice, whereas systemic humoral tolerance was initially induced, but was no longer detected at 6 months[17].

In summary the immunological effects of feeding can be summarised as follows:

1) CMI is usually rapidly and easily tolerised

2) Systemic humoral immunity can be activated, primed or suppressed

3) Secretory humoral immunity is usually activated or primed

These differences between the responses of the humoral and cell-mediated immune responses have been confirmed in many studies. The potential reasons for this split will be reviewed below. In the next section I will discuss various experimental factors which may affect the development of tolerance and in the following section I will consider the mechanisms of tolerance that may account for these differences.

2.3 Factors Influencing the Development of Oral Tolerance

As described above, oral ingestion of antigen may alter the immune responses to that antigen, depending on the study protocol used. A variety of factors, which differed between the studies, seem to be important in influencing the exact immune response seen. These are discussed below.

2.3.1 Type of Antigen

The type of antigen involved can have a profound effect on the mucosal immune response. The most extreme example is when the mucosal immune system is challenged with invasive bacteria such as Salmonella or Shigella species. In this case, a brisk and vigorous immune response occurs, with clear survival benefits to the host.

Cholera toxin is a protein from a pathogenic organism which can promote both a systemic and secretory immune response when given orally[21;22]. Furthermore, cholera toxin can act as an adjuvant to increase humoral responses to other antigens if the two antigens are fed concurrently[21;22]. Cholera toxin can bind to the intestinal cells via a single ganglioside and cause ADP-ribosylation through the cholera toxin subunit-B (CT-B) subunit of the molecule[23]. Both cholera toxin and CT-B can augment the immune response to a conjugated antigen[24]. Others report that coupling CT-B subunit to a second protein can dramatically improve the production of tolerance to that second molecule[25], even in animals that have previously been systemically immunised[26]. The explanation for these different results is unclear.

In contrast to the pathogenic bacteria described above, tolerance can be induced to commensal bacteria[27;28]. Non-bacterial antigens also vary in the ease with which they can induce tolerance. Oral tolerance can be more easily induced by soluble protein antigen than by particulate antigen. One oral dose of soluble antigen can induce tolerance, whereas it is more difficult to induce equivalent levels of tolerance to particulate antigens and larger doses of antigen have to be given[6;14]. Soluble antigens also vary in the ease with which they cause tolerance. For example, in a mouse model 0.25-1mg of myelin basic protein (MBP) will induce tolerance[29], whereas only 0.003mg of type II collagen will have the same effect[30]. It does appear possible, however, to induce tolerance to most proteins if they are given in the right dose and for an appropriate length of time. An exception to this rule is T-independent antigens, which do not induce oral tolerance.

2.3.2 Dose and Feeding Schedule of the Antigen

The dose of antigen is also important. A number of investigators have found that a single, small dose of protein antigen can tolerise CMI[6;17], although, in certain circumstances, very low doses of fed antigen can cause priming of delayed type hypersensitivity (DTH) responses[31]. Larger doses of antigen are required to inhibit humoral immunity[16], and the larger the dose of antigen, the more quickly and completely tolerance is induced[15;32]. The length of feeding also appears to be important. The longer the course of feeding, the more complete the tolerance becomes, both for cell-mediated immunity[33] and for humoral immunity[10;34]. Short courses of antigen feeding may result in priming of the humoral system, but with more prolonged feeding tolerance can occur[14]. As noted above, the different Ig isotypes appear to differ in their susceptibility to tolerance induction. IgE responses can be suppressed by a single low dose of antigen. Larger doses of antigen are required to cause the same effect on IgG and IgA levels[15]. Frequent low dose feeding appears more effective at inducing tolerance than a single, higher dose feed[15;34-36]. The time between oral and parenteral exposure is also important. Using the same dose of antigen, it has been shown that

it is necessary to leave an adequate time interval to allow tolerance to develop (of 4-6 days depending on the antibody isotype studied) before challenging the animal with antigen parenterally[15]. This clearly has implications on how oral tolerance is induced and this will be discussed later.

2.3.3 Immune Status of Animal

Another consideration is whether the animal has previously been exposed to the antigen. Many studies have fed the animal a particular novel antigen before systemic challenge, thus inducing a tolerant state in a naive animal. However the effect of oral feeding on an animal that has already systemically encountered, and therefore mounted an immune response to, a particular antigen is also important. This situation has clinical implications, particularly in considering how oral tolerance might be used to treat established autoimmune disease, a topic which will be discussed later. DTH responses can be suppressed in previously immunised animals[37]. The dichotomy of the humoral response between the various antibody classes becomes even more apparent in previously immunised animals, with IgE levels being (incompletely) suppressed, no effect seen on IgG levels, and IgA levels substantially augmented[15]. Other investigators have examined the effect of dose of feeding on IgG responses in previously immunised animals. They found that early feeding with high dose antigen suppressed the IgG response, whereas lower dose feeding, especially if given later, resulted in priming of the immune response[16]. The dose required to suppress a DTH response also appeared to be higher than in the non-immune animal. Multiple low dose feeds appear to be required to induce oral tolerance in this setting[38;39]. Several reports suggest that the earlier after immunisation that the feeding took place, the more effective the suppression[16;37;38;40]. Thus it appears more difficult to induce oral tolerance in previously immunised animals.

2.3.4 Species of Animal

Another important variable in these various experiments is the species of laboratory animal used. Different species vary in the ease with which tolerance can be induced. For example, rats and mice are very easy to tolerise and often one dose is enough to induce prolonged tolerance[15;17]. On the other hand, it is difficult to induce tolerance in guinea pigs, which require higher doses of oral antigen for longer to become tolerant[10]. Indeed low dose feeding may result in sensitisation of the immune response. Further, there are reports that ruminants, such as cows, or rabbits may not develop significant degrees of tolerance[18;41].

2.3.5 Age of Animal

In addition, the age of the animal appears to be important. For example, in experiments on guinea pigs, Heppell and Kilshaw found that tolerance in cell-mediated immunity was easily and rapidly induced by feeding, but that systemic humoral immunity was increased initially in animals below 3 months of age. With more prolonged feeding however, the systemic antibody level fell compared with non-fed controls, indicating that tolerance was induced in this compartment also. A different picture emerged in animals that were first introduced to the fed antigen when they were older. When feeding was induced at 5-6 months of age, the systemic antibody level rose and remained high despite continued feeding[10]. Other experiments in mice suggest that the mechanisms of tolerance may vary between animals that encounter antigen when they are young and in adulthood. Tolerance appears to be mediated by active suppression in adult mice whereas young animals appear to develop anergy.

The first 7-10 days of life are ones in which oral tolerance may be completely absent: feeding neonatal mice an equivalent weight-dose of OVA as adults resulted in priming of both humoral and cell-mediated immune (CMI) responses, which is a very unusual response in adults[36]. It was initially suggested that an immature immune system was unable to process antigens so as to induce tolerance. However, further work has suggested that this may be due

to a regulatory imbalance since the failure to induce oral tolerance can be partially restored with adult spleen cells[42].

Weaning is another period of early life when oral tolerance appears to be incomplete[43] and is independent of the time at which weaning occurs. Interestingly, there are major changes in the gut immune system at the time of weaning[44], which suggests that there may also be a regulatory cell imbalance at this time.

2.3.6 The Presence of Commensal Bacteria

It has been demonstrated that oral tolerance cannot be induced to sheep red blood cells in germ free mice but can in normal mice of the same strain[45]. In other studies, tolerance to soluble antigen can be induced in germ free mice, but the duration of tolerance is shorter[46]. In addition, the feeding of lipopolysaccharide can enhance the induction of oral tolerance[47] and induce tolerance in germ-free mice[45]. Thus the presence of commensal bacteria in the intestine, or at the very least immunogenic bacterial products, seems to be important to the induction of tolerance.

2.4 Mechanisms of Oral Tolerance

2.4.1 Introduction

Advances in cellular and molecular biology have improved understanding of the control of immune responses and allowed the mechanisms by which tolerance is induced to be investigated. In this section I will outline the various proposed mechanisms of tolerance production and review the evidence in the literature on how these potential mechanisms apply specifically to oral tolerance. Firstly however, a brief review of the mechanisms of induction of an active immune response is included, since an understanding of these is required to appreciate the theories of tolerance production.

2.4.2 The Generation of an Immune Response – a Dual Signal Process

Immunocompetent cells can recognise specific antigens. B cells do this via their surface Igs and T cells do so via the T cell receptor (TCR). Both of these interactions are antigen specific and the occupation of these receptors is necessary for cellular activation, but importantly it is not enough to do so alone. A second signal is necessary to complete the activation process[48;49]. In the case of T helper cells this can be provided by soluble interleukin (IL) -1 produced by activated macrophages or T cells[50;51]. Others suggest that cell-to-cell contact is required to provide the second signal[52]. Surface molecules on antigen presenting cells (APCs), such as the B7 family of molecules, may provide the second signal in this case[53;54].

Once activated, T helper cells can then provide the second signal, or "help", to antigen specific cytotoxic T cells via IL-2, and provide help for B cells by producing cytokines IL-4 and IL-5. These cells are therefore activated if they also encounter antigen at the same time. All of these cytokines act in a paracrine manner. The cells involved are grouped close together in areas of lymphoid tissue such as lymph nodes, which create the optimal conditions to produce an efficient immune response.

It has been noted that most B cells require "help" from T cells. One exception to this rule is that certain T-independent antigens can stimulate B cells without T cell help. These antigens are polyvalent and cross-link surface Ig on B cells which provides enough stimulation to activate B cell without T cell help. Typically these antigens are bacterially derived and include peptidoglycan from bacterial cell walls and single-stranded RNA. It is interesting that these antigens do not induce oral tolerance, which does suggest that T cells are central to the production of oral tolerance.

A further level of complexity is added by the theory that activated B cells can conversely give help to T cells. Activated B cells can provide this help, by acting as APCs, presenting antigen bound to their surface Ig, and providing a second signal. On the other hand, resting B cells, although they can present antigen via their surface Ig, do not provide co-stimulation[49;55]

and therefore do not provide help to T cells. It may be that activated B cells express high levels of B7, a potent co-stimulatory molecule, on their surface[56]. According to this theory, B cells are crucial in the outcome of an immune response, and provide an important control mechanism to prevent an inappropriate immune response.

2.4.3 T helper Cell Subsets

From the above section it can be seen that T helper cells have a crucial role in the generation of an immune response. There is now ample evidence that in the mouse model T helper cells can be divided into at least two distinct subsets on the basis of the cytokine profile that they secrete and the effector functions that they mediate. These cells have been termed T_H1 and T_H2 cells.

2.4.3.1 T_H1 cells -T_H1 cells secrete IL-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α[57]. These cells seem to induce preferentially a CMI response and increase DTH. IL-2 and IFN-γ are major factors which supply help to cytotoxic T cells. In addition it seems that only T_H1 cells can cause a delayed-type hypersensitivity (DTH) response, and that T_H2 cells are incapable of producing this type of immune response[58]. The response of B cells to T_H1 help is variable. Some studies suggest that T_H1 cells are incapable of stimulating resting B cells[59]. Others show that IL-2 appears to be able to stimulate B cell proliferation and antibody production *in vivo*. It seems T_H1 cells can increase the IgG2a antibody response through the secretion of IFN-γ[60], and this cytokine also appears to inhibit other Ig subclass responses such as IgG1, which may be induced by T_H2 cytokines[59]. Thus IFN-γ may have a role in Ig isotype switching.

2.4.3.2 T_H2 cells - T_H2 cells secrete IL-4, 5, 6, and 10[57]. These cells and cytokines seem particularly adept at stimulating a humoral response[59]. IL-4, IL-5 and cell to cell contact are

required for optimum resting B cell proliferation and differentiation[59]. The secretion of certain Ig isotypes are particularly enhanced by T_H2 cells, particularly IgA, IgE (for which IL-4 help appears to be of crucial importance) and IgG1[60]. In addition IL-4 and IL-5 can provide help for cytotoxic T cells[61], but not for delayed type hypersensitivity (DTH)[58]. Indeed, IL-4 and IL-10 act in synergy to inhibit a DTH response induced by T_H1 cells[62]. Lastly, until the recent discovery of eotaxin[63], IL-5 was the sole known chemo-attractant for eosinophils and causes eosinophil proliferation, and thus a T_H2 response may also be important for developing an immune response against parasitic infections.

Thus the type of immune response to an antigen can vary depending on the type of T helper cell responses produced. A T_H1 response will produce a vigorous cell-mediated immune response, but a poor to moderate humoral response, whereas a T_H2 response will have the opposite effects.

2.4.3.3 Differentiation of T_H1 and T_H2 Cells

How does this differentiation into T_H1 or T_H2 cells occur? Before exposure to antigen, CD4+ T cells appear to secrete IL-2, but only minimal levels of other cytokines[64]. After short-term culture, T helper cells can express many cytokines, including IL-2, IL-4, IFN-γ and TNF[64]. Many T cells obtained directly from experimental animals show this pattern of lymphokine secretion[65]. Only after more prolonged culture do cells show the typical pattern of T_H1 or T_H2 cytokine secretion discussed above. Cell clones derived from single cell precursors can be induced to secrete either IL-2 or IL-4, depending on the cell culture conditions[66;67]. These results have lead to the theory that there is an immature precursor T helper cell, termed T_Hp. After short term culture this cell differentiates into a cell which can secrete many cytokines, including IL-2, IL-4, IL-5,IL-10 and IFN-γ. This cell has been termed the T_H0 cell[66;67]. With prolonged exposure to antigen, this cell is then thought to differentiate into either a typical

T_H1 or T_H2 phenotype, depending on prevailing conditions at the time of antigenic stimulation[64].

The cause of this switch appears to be the fact that naive T cells will switch on and off appropriate genes. It seems that those cells which become T_H1 cells switch off their IL-4 genes, whereas those that become T_H2 cells extinguish their IFN-γ genes[68]. Conversely those that become T_H1 cells up-regulate their IFN-γ genes and those that become T_H2 cells up-regulate their IL-4 gene. This process is not detectable at 20 hours, but is present at 40 hours[67].

2.4.3.4 Factors Influencing the T_H1 To T_H2 Switch

The above observations lead to the question of which factors influence the outcome of this differentiation pathway? It has been observed that the overall immune status of the animal will influence the proportion of T_H1 to T_H2 cells found in subsequent culture. In particular, if the animal is immunised with an antigen that normally induces a profound T_H2 antigen-specific response, the overall number of T_H2 clones in subsequent culture is greatly increased and the speed with which they develop is also increased. Conversely immunisations that induce a T_H1 antigen-specific response increase the overall number of T_H1 clones observed[64]. These results suggested that *in vivo* factors could influence the outcome of the immune response.

In *in vitro* culture it has been observed that the culture conditions can profoundly influence the type of T cell proliferation seen. Culturing lymphocytes with IL-4 resulted in increased levels of IL-4[69] and reduced/unchanged levels of IL-2 being produced[70]. Adding IFN-γ or anti-IL-4 antibodies to the cell culture resulted in reversal of the pattern of cytokines production[69-71]. IL-10 has also been shown to inhibit cytokine production from T_H1 cells[72]. It may be that, at least in part, IL-10 exerts its action by an effect on macrophages such that

when they present antigen they do so in such a way that inhibits a T_H1 response[72]. Interestingly IL-10 and IL-4 may act synergistically to down-regulate T_H1 functions[62].

Conversely, if culture medium contains IFN-γ, T_H2 cells are inhibited whereas T_H1 clones are unaffected, suggesting that this cytokine will promote a T_H1 response[73;74]. IL-12, another pro-inflammatory cytokine, also promotes a T_H1 response[68]. Therefore, the cytokine milieu present at the time of the induction of the immune response may critically influence its outcome by altering the T_H1 to T_H2 helper cell ratio.

The length of time in culture also affects the type of T helper cell produced. At an early stage in their culture, T helper cells respond to IL-2 and IL-4 in a synergic manner. After more prolonged culture, T_H1 clones become more responsive to IL-2 and less responsive to IL-4 than T_H2 clones[74]. It is possible that early on these cells are not fully differentiated (i.e. T_H0 cells) and are thus able to respond to both cytokines. With more prolonged culture they differentiate terminally into, and demonstrate the more classical features of, the individual subsets of the T helper cell subsets. The polarisation of the immune response seems to become more pronounced with time, so that chronic immune responses becomes progressively more directed towards either a T_H1 or T_H2 response. On the other hand short duration immune responses may be able to show a mixed pattern since the typical T_H1/T_H2 response only appear with time as these polarised responses develop.

Although the cytokine milieu at the time of antigen presentation seems to be the most important factor influencing the T_H1/T_H2 switch, other factors also seem to be important. These include the type of antigen, the route of antigen entry and the dose of antigen[75]. Clearly if the oral route of antigen administration results in the production of T_H2 cells, then a degree of tolerance, at least for CMI, by immune regulation will be achieved. The evidence that orally administered antigen can result in tolerance by this mechanism will be discussed below.

2.4.3.5 Host Benefits of the T_H1/T_H2 Paradigm

It has been observed that many immune responses to infectious organisms are either predominantly cell mediated or predominantly humoral. The type of immune response that is best suited to clearing a particular organism depends on the type of organism involved. For example cell-mediated immune responses are better able to eradicate intracellular organisms, whereas extra-cellular organisms are best countered by a combination of cell-mediated and humoral immunity. Lastly, metazoan infections are best countered by a humoral immunity with no cell-mediated immune response since the latter may cause more damage to the host than the organism. The T_H1/T_H2 responses have clearly evolved to allow this dichotomy of immune response to occur[76;77]. Most antigens encountered in the gastrointestinal tract would be best dealt with by a strong humoral immune response. This will bind toxins and prevent binding of bacterial to the mucosa by coating any binding proteins. This strategy will keep noxious substances in the gut lumen. Conversely, a strong cell-mediated immune response will be potentially harmful to the host, since it may damage the gastrointestinal mucosa. Thus it can be seen that the most appropriate response to antigens in the gastrointestinal tract would be a T_H2 response. The evidence that this occurs and may account for oral tolerance is reviewed below.

2.4.3.6 Evidence for T Helper Cell Subsets in Humans

Much of the above work has been done in mice. Studies of clones of human helper cells have identified clones that correspond broadly with the T_H1 and T_H2 phenotypes observed in mice. For example it was shown that purified protein derivative (PPD) specific T cells clones secreted IL-2 and IFN-γ, which are typical of T_H1 cells. Conversely T cell clones specific for secretory-excretory antigen of Toxocara canis produced IL-4 and IL-5, which is a pattern of T_H2 cells. These cells persisted with stable cytokine profiles over 6 months in culture[78]. Further, the cells appeared not to secrete the opposing cytokines because they lacked

transcripts for these cytokines, which provides indirect evidence that the genes for these cytokines were switched off.

In other studies, there were in addition many other clones which seemed to produce a mixed pattern cytokine profile[79]. All groups of clones could provide help to B cells producing IgG, but only IL-4 producing clones (i.e. T_H2 phenotype clones) could provide help for IgE producing B cells[79]. It has been suggested that human cell cultures persist for longer as a mixed phenotype before maturing down one development pathway than do mouse cultures[80]. This suggests that a mixed T_H1 and T_H2 response may occur for longer and more easily in a human immune response than in the murine model.

Lastly, evidence from the immune responses to specific pathogens, or in autoimmune disease, fit into a predominantly cell-mediated, T_H1 type response or a predominantly humoral, T_H2 type response[76], in exactly the same way as happens in animal models. This provides further circumstantial evidence for the existence of T_H1 and T_H2 cell types in humans.

2.5 General Mechanisms of Tolerance

With this background into the production of an immune response, it becomes possible to postulate various mechanisms of tolerance induction and maintenance. The hypotheses that have been suggested to explain the principles of tolerance in general will be discussed in this section. In the following section the evidence that these general mechanisms of tolerance production can be applied to oral tolerance will be discussed.

2.5.1 Immune Deviation

As noted above, the immune response can to be divided into a T_H1 or T_H2 response[57;58]. The two responses produce different immune profiles and work to suppress the effects of the other type of T helper cell[57]. Thus, the production of a T_H2 response can be thought of as producing suppression of cell mediated immune responses, and therefore tolerance of this

limb of the immune response to that antigen[58]. This mechanism, termed immune deviation, does not produce tolerance by the strict definition of the term, but the alteration of the immune response produced may have a beneficial effect on the host. Thus, in situations where a cell-mediated immune response may be damaging to the host, the production of a T_H2 cell allows a degree of active immunity while at the same time protecting the host from an excess immune response[77;78]. It seems to occur in response to specific infections, especially parasitic[81]. It also has been suggested as the reason why the foetus is not rejected by the systemic immune response[76]. Evidence that orally encountered antigen causes immune deviation will be discussed below.

2.5.2 Active Suppression

Another mechanism of tolerance that has been put forward is active suppression. It has been suggested that there is a sub-population of antigen specific cells that when activated secrete specific suppressive cytokines. According to this theory these cells, when activated, will down regulate an active immune response through the actions of these suppressive cytokines. It is suggested that the mechanism of antigen presentation in the gut favours the production of suppressive immunoregulatory cells over cytotoxic (and possibly even antibody forming) cells. Many early investigators postulated that this is the mechanism by which oral tolerance is induced[11;34;82], but it is only in recent years that direct supportive evidence has been uncovered. Even now the nature of these cells and cytokines is uncertain and, as will be discussed below, CD4+ and CD8+ cells may both act as suppressor T cells[83-85].

2.5.3 Clonal Anergy

Another mechanism of tolerance is "clonal anergy", which has been reviewed by Schwartz[86]. This mechanism is based on the dual stimulation theory of lymphocyte activation. One stimulus is from the antigen itself. B cells recognise the whole antigen via their surface Ig, whereas T cells recognise antigen fragments in combination with MHC antigens via their T

cell receptor. The second signal may be from soluble mediators, or from cell surface molecules which require cell to cell contact to be activated. This has been reviewed in the section on generation of an immune response.

Crucially, without this second signal, B cells do not produce antibody[49] and T cells do not proliferate or produce IL-2 in response to subsequent antigen exposure, even in conditions appropriate for immune activation[87;88]. These lymphocytes have been rendered anergic. Anergy is also suggested by the observations that B cells down-regulate the expression of surface antigen and that T cells down regulate their surface IL-2 receptor[55].

The encounter of the T cell with antigen in the absence of co-stimulation does not appear to be a purely negative event since the cells increase in size and IL-3 production is increased in cells that encounter antigen in this way before they become unresponsive[88]. This suggests that the initial encounter with the antigen causes partial activation with intra-cellular signalling followed by down regulation and anergy.

The need for the second signal in the activation of an immune response appears to be an important control on the immune system. It ensures that an immune response is only stimulated in the presence of inflammation, and thus may prevent an immune response being generated to harmless or self antigens. This is best illustrated by the fact that self-reacting B cells have been identified and it is thought to be lack of co-stimulation that prevents them from producing auto-antibodies[49].

Clonal anergy can develop in the thymus[89], or to antigens given intravenously or intraperitoneally[90]. This review will discuss the evidence that oral administration can result in the induction of anergy. It has been postulated that in the environment of the gastrointestinal mucosa, antigen is presented in an atypical manner which leads to anergy[91-93]. Thus immunocompetent cells which recognise dietary antigens may become anergic and unable to mount a systemic immune response in future.

2.5.4 Clonal Deletion

A fourth mechanism of tolerance induction is clonal deletion. This mechanism is particularly important in generating tolerance to self-antigens. The major site of clonal deletion occurs in the thymus as T cells are maturating. Here the T cells undergo rearrangement of their T cell receptor (TCR) genes by a combination of splicing and mutations. The products of these genes - i.e. the T cell receptors themselves - are then expressed. Thymocytes express major histocompatibility complex (MHC) antigens. The newly formed TCRs come into contact with these MHC antigens. If they bind to them too strongly, they are likely to do so outside the thymus and thus possibly induce an autoimmune reaction. Conversely if they bind them too weakly, they will never be able to participate in an immune response even if the MHC molecules present an antigen fragment. Therefore, if the TCRs bind MHC too strongly or too weakly in the thymus, that cell is induced to undergo apoptosis or "programmed cell death". It is only if they fall in the useful middle ground that they receive the required stimulus to continue to mature and eventually leave the thymus[89;94].

As can be seen from the above brief review, much of the work on clonal deletion suggests that it occurs in the thymus and is responsible for maintaining self-tolerance. For a long time it was felt that clonal deletion did not occur in the periphery[94]. Recent evidence suggests that this may not be the case, and clonal deletion can occur in response to oral antigens. The evidence for this will be discussed below.

2.6 Immune Mechanisms of Oral Tolerance Production

Evidence exists to support a role for all of these mechanisms in the induction of tolerance, and it is likely that they all are involved, either singly or together under different circumstances.

2.6.1 Clonal Deviation

This subdivision of T helper cells into T_H1 and T_H2 subtypes offers a potentially useful explanation for the observations that the different limbs of the immune response behave differently in response to oral antigens. Cell mediated immune responses are frequently suppressed, but the humoral responses can be elevated, primed or suppressed. It may be that the presentation of antigens via routes that induce tolerance results in a predominately T_H2 response thus skewing the immune reaction towards a humoral response and reducing the cell mediated effects, particularly DTH. This hypothesis is supported by the observation that the antigen specific T_H2 mediated IgG1 production is maintained whereas the T_H1 mediated IgG2a response is inhibited by feeding of that antigen[95]. The humoral response would have the benefit of binding any bacterial toxins or coating bacterial binding sites in the gut lumen and thus exclude potentially harmful antigens from the body. At the same time, the reduction of the cell-mediated immune reaction may protect the gut from a damaging inflammatory response to harmless antigens. Thus the T_H2 cells may be acting as active suppressor cells (see below).

2.6.2 Evidence for Active Suppression in Oral Tolerance

2.6.2.1 Cyclophosphamide

Early evidence for the existence of suppressor T cells was derived from the observation that pre-dosing animals with cyclophosphamide abrogated the subsequent attempts to induce oral tolerance. Evidence that cyclophosphamide selectively affects T suppressor (T_S) cells is quoted from both *in-vitro*[96], and *in vivo*[97;98] work. These studies all suggest that at doses of about 100 milligrams (mg)/kilogram (kg)/mouse, cyclophosphamide up-regulates the immune response by inhibiting T_S precursor cells.

This work was extended to oral tolerance where cyclophosphamide has been shown to prevent the induction of oral tolerance if given to mice before feeding occurs. An experiment

in which mice were fed high or low dose OVA before immunisation showed that high dose feeding resulted in tolerance in systemic CMI and serum IgG and IgM responses whereas low dose feeding resulted in tolerance in systemic CMI and, to a lesser extent, serum IgM responses. Pre-treating these animals cyclophosphamide, given intra-peritoneally, resulted in the complete blockage of tolerance induction in the low dose fed group, whereas that of the high dose group was only partially blocked[99]. These results support the idea that the separate arms of the immune system are controlled differently, as suggested by the evidence discussed above. Further it may be that low dose feeding induces T_S cells which act primarily on cell mediated responses, and that it is these responses that are inhibited by cyclophosphamide. As will be discussed below, perhaps higher dose feeding induces different mechanisms that may not be disrupted by cyclophosphamide.

2.6.2.2 Transfer Experiments

The ability to transfer tolerance from an animal that has been fed a specific antigen to a naive animal is a further powerful line of evidence that active suppression occurs, and many of the studies quoted used transfer experiments to help elucidate the mechanisms of tolerance. Many early experiments showed that transfer of cells from the Peyers' patches, peripheral lymph nodes or spleens of pre-fed animals could transfer tolerance to naive control animals[6;11;13;32;34;82;100].

In addition some authors suggested that transfer of serum could transfer tolerance. There appear to be two situations in which serum can transfer tolerance. Firstly, it occurs when serum is taken two weeks after the commencement of feeding[101] and the tolerogenic factor may be antigen-antibody complexes. Other authors report failing to transfer tolerance with serum taken at this time[33]. The second situation in which serum could transfer tolerance was when serum was taken one hour after a single feed of antigen such as OVA[102]. The factor responsible for the transfer of tolerance seemed to be OVA itself, since the ability to transfer tolerance was removed if the serum was treated with Sepharose beads coated with anti-OVA

antibody, thus removing OVA from the serum[103]. These experiments suggest that processing antigen by the gastrointestinal immune system is crucial in determining tolerance. These ideas will be discussed in detail later in this thesis.

In the majority of the studies examining cell transfer, it was felt that the suppressor cell was a T cell since the cells were susceptible to anti-rat lymphocyte serum[100] or anti-Thy 1.2 antibodies and complement[11;82]. In at least one experiment, it was suggested that B cells could also transfer tolerance since these cells rosetted antibody/complement coated red blood cells and their resistance to anti-θ serum plus complement[13]. Other authors have been unable to reproduce this finding[82]. The suppressor T cells were initially found in Peyers' patches and, after 1-2 days, in the peripheral lymph nodes[20;100]. After 4 days however, cells from these sites were no longer able to transfer tolerance, but cells from the spleen or thymus could[100]. These findings suggest that suppressor cells migrate from the gut, where they are presumably induced, to more distant lymphoid sites. Other models give similar results that agree with this hypothesis[13].

All the researchers that investigated this subject found that transfer was always antigen specific, which is further evidence that oral tolerance is antigen specific. A further feature of the transfer of tolerance was that it appeared to affect the afferent limb of the immune response since transfer of cells had to take place before or immediately after systemic challenge of the recipient animal with the antigen to transfer tolerance[32;33].

2.6.2.3 Bystander Suppression

Another observation that added weight to the theory that active suppression was involved in oral tolerance was termed "bystander suppression". In experimental animals an active immune response was evoked to an antigen and oral tolerance was induced to an unrelated antigen. When the antigen to which an active response had been raised was added to a cell culture system, cell proliferation was seen. No proliferation was seen when the tolerising

antigen was used (as would be expected). When both antigens were added to the same system, no response was seen to either antigen[83]. Thus some active component induced by the tolerising antigen must be suppressing the active immune response - causing bystander suppression. Another example of bystander suppression comes from the observation that an animal made orally tolerant to a protein that is subsequent injected linking to a hapten does not produce an immune response to either antigen or hapten[32]. More recent reports by the same group and others have confirmed the presence of bystander suppression[104;105]. It is suggested that cytokines released by suppressor cells produced by orally feeding antigen can act in a non-specific manner to down-regulate all immune responses, provided that the suppressor cells are activated in an antigen specific manner initially.

Other investigators have used conjugated antigens such as dintrophenylate (DNP)-OVA. Animals were made orally tolerance to OVA. Immunisation with DNP-keyhole limpet haemocyanin (KLH) resulted in good immune responses to DNP being generated, whereas if DNP-OVA was injected then the immune response to DNP was reduced. Further if DNP-KLH and DNP-OVA were injected together, even if the injections took place at different sites or at different times, the immune response to DNP was reduced[106]. These authors argue that since this suppression seems to operate over a larger distances and times, the cytokine mediated theory for bystander suppression cannot fully explain their observations. They postulate than an idiotypic network theory of immune response may be better able to explain their results.

Finally, a more recent suggestion is that bystander suppression may be mediated by the down regulation of APCs that are then unable to present antigen and provide help for CD4+ cells recognise unrelated antigen[107;108].

2.6.2.4 The Identity Of The Suppressor T cell

The nature of the T suppressor cell has caused considerable controversy. Many workers have identified it as a CD8+ cell[83;109-111]. However, others report that CD8-depleted animals could

still develop oral tolerance[112-114], although there was reduction of local tolerance, as manifest by normal mucosal IgA production, in CD8 deficient mice[114]. In other models, CD4+ cells were the mediators of oral tolerance[84;115;116]. Depletion of CD4 cells with anti-CD4 antibodies prevented tolerance induction[84] and a CD4+ deplete strain of mouse did not develop tolerance[117]. One report suggested that both CD4+ and CD8+ cells could transfer tolerance separately in one experimental model[112].Thus it seems that both CD4+ and CD8+ T cells can act to mediate oral tolerance in different models, and possibly are complementary to each other in maintaining tolerance.

Other reports suggest that γδ T cells may be important in the production and maintenance of oral tolerance. They demonstrated that the production of tolerance could be blocked by the use of an anti-γδ TCR monoclonal antibody, in both *in vivo*, and *in vitro* transfer experiments[118;119], and one strain of γδ cell knockout mice do not develop oral tolerance[119]. Furthermore adoptive transfer of γδ cells from animals fed peptides fragments of S-ag ameliorated experimental autoimmune uveitis[120]. These reports are not consistent with other experimental models which suggest that γδ T cells act as T helper cells or counter-suppressor cells and it has been suggested that γδ T cell subsets can have different functions depending on the prevalent conditions at the time of activation. Finally others report that γδ T cell deficient mice have a reduced IgA response to oral antigen, leading these authors to postulate that γδ cells have a specialised role in controlling mucosal immunity[121].

2.6.2.5 The Role of Cytokines in Active Suppression

The above evidence suggests that, when animals are fed oral antigen, suppressor cells are induced. The next question is how do these cells exert their action? It is clear that antigen specific triggering of these cells is required to induce tolerance, since antigen specificity is an important feature of oral tolerance that has been identified in many studies as discussed above. Once triggered, suppressive soluble mediators or cytokines may be secreted. For

example it has been shown that if suppressor cells and effector cells are separated by a semi-permeable membrane, which prevents cell-to-cell contact, suppression of the effector cells still occurs and this must be by the presence of a soluble mediator. Further, these soluble mediators have been shown to induced bystander suppression using this same system[83].

2.6.2.6 The Nature of the Suppressive Cytokines

2.6.2.6.1 T_H2 Cytokines –Evidence for Clonal Deviation

Thus there is plenty of evidence that suppressive factors are involved in tolerance. The next stage was to identify them. Theoretically the immunological effects of oral tolerance in many models, including the suppression of CMI and the priming of humoral immunity, could be mediated by switching the T helper response towards the T_H2 type response. Evidence to support this hypothesis came later when cytokine analysis became possible. Examples of this type of cytokine work come from experimental models of autoimmune disease, where the primary end-point of the effectiveness of oral tolerance is a reduction in the observed severity of that disease. For example, non-obese diabetic (NOD) mice become diabetic spontaneously at about 15-20 weeks of age. The disease is characterised by an inflammatory infiltrate of CD4+ T cells that secrete T_H1 type cytokines into the pancreatic islets. Feeding these mice insulin from 5 weeks of age reduces the insulitis and abrogates the disease. Cytokine analysis showed increased expression of IL-4 and IL-10 which are T_H2 cytokines, and transforming growth factor (TGF)-β. There was a parallel fall in the T_H1 cytokines IL-2, IFN-γ and TNF-α[122]. Other authors report similar findings. For example, feeding myelin basic protein induced a T cell that produced IL-4 and IL-10 (i.e. T_H2 type cytokines) and inhibited T_H1 induced autoimmune encephalitis[85]. Another report shows that feeding of OVA caused increased IL-4 messenger ribosomal nucleic acid (mRNA) expression, but reduced IFN-γ mRNA expression[123]. Taken together, these results suggested that oral

tolerance could occur by switching the immune response from a harmful T_H1 response towards a safer T_H2 response.

Further evidence that a switch from a T_H1 to a T_H2 based response may be important in oral tolerance is supported by the finding that some of the effects of oral tolerance could be inhibited by the administration of anti-IL-4 antibodies at the time of tolerance induction[124], and giving IL-4 itself at the time of tolerance induction can enhance oral tolerance[125]. As discussed above, IL-4 is a T_H2 cytokine that can inhibit the production of $T_H1$cells. Therefore blocking IL-4 may influence the balance between the ratio of T_H1 to T_H2 cells, resulting in an increased T_H1 response, and thus abrogate tolerance. Other studies have looked at the effect of giving pro-inflammatory cytokines to orally tolerant animals at the same site and time as an immunising injection. They found that IL-12 was able to reverse tolerance, although IL-2, IFN-γ and GM-CSF could not[126]. Others have found that giving IL-12 neutralising antibodies could promote oral tolerance[127], which again suggests that IL-12 may inhibit oral tolerance. IL-12 may mediate its effect in part by changing the local immune environment such that T_H1 responses are promoted and T_H2 type responses inhibited.

Other observations do not fit with the theory that oral tolerance may be mediated by immune deviation towards a T_H2 type response. For example, IgE responses are often dramatically reduced by oral feeding[15;20], and yet IgE is regarded as a T_H2 mediated response. Also tolerance to intranasal antigen can be induced even in the presence of anti-IL-4 antibodies, which should inhibit T_H2 responses[128]. Furthermore peripheral tolerance can be induced by feeding in STAT6 knockout mice[129]. STAT proteins are deoxyribonucleic acid (DNA) binding proteins involved in cell signalling. STAT6 is involved in IL-4 signalling and thus STAT6 deficient mice are functionally deplete of T_H2 cells. Oral tolerance is also normal in IL-4 knock out mice[128;130]. These results suggest that tolerance can be mediated in the absence of T_H2 cells. Although oral tolerance was induced by feeding a large dose of antigen, and therefore may be mediated by clonal anergy (see below), it has been suggested

that other cell type(s), related to but distinct from T_H2 cells, may be the mediator of oral tolerance.

2.6.2.6.2 T_H3 Cells and Transforming Growth Factor-β

Other work has suggested that different cytokines may be important in oral tolerance production. One such cytokine is TGF-β. Activated T cells[131] and B cells[132] appear to have the ability to secrete TGF-β. *In vitro* work suggests that TGF-β can inhibit IL-2 dependent T cell proliferation and suppress B cell proliferation and antibody secretion[131]. Thus, TGF-β seems likely to be an important suppressive cytokine *in vivo*. Levels of this cytokine are increased in many models of tolerance[29;133;134]. Further neutralising antibodies to TGF-β prevent the production of tolerance by effector cells[115;135;136]. The cells that secrete TGF-β were found to be CD4+ cells in the majority of these reports.

Thus it may be that the production of antigen specific TGF-β secreting cells by the gastrointestinal immune system is an important mechanism of tolerance induction. Supportive evidence that this may be the case comes from experiments that have shown that feeding more prolonged doses of antigen results in a fall in both T_H1 and T_H2 cytokines, whereas TGF-β levels rise[133]. The authors suggested that this may represent a specific cell type with suppressor properties and they have suggested that this cell be termed the T helper 3 (T_H3) cell[133]. Similar TGF-β secreting antigen specific cells have been detected in humans fed myelin[137]. It has been reported that these cells also need IL-4 as a growth factor[125], which may explain why anti-IL4 can inhibit oral tolerance as discussed above. Therefore TGF-β secreting T_H3 cells may be responsible for inducing oral tolerance in some experimental models.

It should be noted that in at least one experimental model using TGF-β1 knockout mice oral tolerance could still be induced[138] and therefore there must be other mechanisms of tolerance induction in this model.

2.6.2.6.3 T_R1 Cells and IL-10

Recently a further population of regulatory T cells have been identified by repeatedly stimulating OVA specific naïve CD4+ cells obtained from αβ T cell receptor transgenic mice[139]. These cells secrete high levels of IL-10 and IL-5, some TGF-β and IFN-γ, proliferate poorly in response to antigen stimulation and have been termed T_R1 cells[139]. Cells secreting a similar T_R1 cytokine profile could also be generated from human blood if stimulated with APCs and IL-10[139]. IL-10 is a potent suppressor of T_H1 cell functions and can inhibit T_H1 mediated disease such as IBD *in vivo*[139]. These properties are similar to the effects observed in studies on oral tolerance and have led to speculation that such cells may be induced by encounter of antigen at the gastrointestinal mucosa.

2.6.2.7 Summary of Work Suggesting that Suppressor Cells May Play a Role in Oral Tolerance

There is thus good experimental evidence, principally from transfer experiments, that suppressor cells exist and are important in mediating oral tolerance. Further work has suggested candidate suppressor cells and these include T_H2 cells, which can suppress the T_H1 cells, T_H3 cells which produce TGF-β and may act as more generalised suppressor cells and possibly T_R1 cells that modulate the immune response principally through the secretion of IL-10. In addition CD8+ T cells have been shown to transfer tolerance, and may do so by producing a similar array of cytokines[140], although the evidence that this occurs in the context of oral tolerance is so far lacking.

2.6.3 Evidence for Clonal Anergy in Oral Tolerance

Initially most experimental studies suggested that suppressive mechanisms were the cause of the observed tolerance for the reasons outlined above. Experimental evidence that this was not always the case started to emerge.

2.6.3.1 Failure to Transfer Tolerance

Whitacre et al developed an experimental system using experimental autoimmune encephalitis (EAE) in rats[141]. They demonstrated that feeding myelin basic protein resulted in decreased clinical signs when these mice subsequently had EAE induced. They were unable, however, actively to transfer tolerance using cells derived from the spleen, lymph nodes or Peyers' patches or indeed using humoral elements. Further these mice were not protected from EAE when lymphocytes from other non-fed, diseased mice were transferred into them. Both these observations suggest active suppression did not occur in this experimental system. They also showed that lymphocytes derived from the spleen and lymph nodes of pre-fed animals had a reduced proliferative response *in vitro* and produced less IL-2 when stimulated. As discussed, these are both features of anergic cells, and Whitacre *et al* argued that this might be the explanation for the type of tolerance they were seeing in their system. Furthermore they reported that changes in one amino acid in a tolerogenic peptide dramatically reduced the ability of that peptide to protect against EAE compared to when the original peptide was used. Thus there appeared to be exquisite peptide specificity when inducing oral tolerance which they again suggested implied that clonal anergy rather than bystander suppression was the mechanism of tolerance induction[142].

Finally, another group of investigators demonstrated that oral tolerance to OVA could be induced in mice pre-treated with cyclophosphamide[143]. Tolerance could not be transferred by cells obtained from pre-treated animals. The authors argued that cyclophosphamide destroyed T suppressor cells and therefore tolerance could be induced by means other that active suppression.

2.6.3.2 Reversal of Tolerance With IL-2

The findings discussed above have been confirmed[144], and extended to show lack of bystander suppression both *in vivo* and *in vitro*. The cells also showed reduced ability to produce IL-2 receptors in addition to reduced IL-2 secretion, both of which are features of anergic cells. In addition, pre-culturing peripheral lymph node cells with IL-2 resulted in a return of the ability to proliferate in response to fed antigen demonstrated *in vivo* and *in vitro*, which suggested that anergy was being overcome by this powerful stimulus[144;145].

2.6.3.3 Direct Evidence for Clonal Anergy as a Mechanism of Oral Tolerance

The above results strongly suggest that clonal anergy can be responsible for oral tolerance production. Direct evidence for the presence of clonal anergy was derived from observations on OVA specific TCR transgenic lymphocytes that were transplanted into mice. These mice were fed OVA and the effects on the transgenic lymphocytes were monitored. It was shown that these lymphocytes were not deleted, but that they had reduced proliferative responses following feeding. This did not appear to be due to suppressive factors and the authors concluded that the effects were due to clonal anergy[146]. Further studies suggest that clonal anergy is not a passive process, since cells that encounter antigen presented orally show an increase in size and a transient up-regulation of IL-2 production and expression of activation markers such as CD69[147]. Subsequently, there is a marked reduction in IL-2, IL-10 and IFN-γ production and a failure to respond to antigen *in vitro*[147]. These results suggest that there is active differentiation of antigen specific T cells towards the anergic state when antigen is encountered orally.

2.6.3.4 T Helper Cell Subsets and Clonal Anergy

Further work suggested that the subsets of T helper cells vary in their susceptibility to anergic stimuli. T_H1 cells appear to become anergic more readily than T_H2 cells, with lower doses of antigen being required[148;149]. This suggests that clonal anergy of T_H1 cells after antigen feeding may allow T_H2 immune responses to be manifested further without competition from T_H1 cells. This also led to the hypothesis that both clonal anergy and immune deviation may work together to enhance oral tolerance. Feeding high dose of antigen, however, causes both T helper cell subsets to be rendered anergic[130].

2.6.4 Evidence for Clonal Deletion in Oral Tolerance

Clonal delction is thought to be the main mechanism for generating self-tolerance. It is known to occur in the thymus during maturation of the immune system. For many years, it was thought not to occur in the mature immune system. Recently, however, there has been increasing evidence to suggest that clonal deletion can occur in the periphery. It has been shown in transgenic mice cloned for a particular TCR that recognises OVA, that T cells numbers fall in the Peyers' patches in response to high dose feeding of 50-500mg per dose of OVA. This is not due to a migration of these cells to other lymphoid or non-lymphoid organs. In addition, increased apoptosis was observed in the Peyer's patch at the time of this fall in numbers suggesting that "programmed cell death" is responsible for the loss of T cells[133]. This did not occur when these animals were fed bovine serum albumin (BSA) suggesting that this mechanism is antigen specific. In addition, the fall was not observed when non-transgenic mice were fed OVA[133]. Low dose feeding (0.5mg/feed) failed to cause any measurable clonal deletion even if the length of feeding was extended to a month[133]. Other authors have confirmed the finding of clonal deletion in the OVA TCR-transgenic mouse[127].

How does clonal deletion occur? Apoptosis is a common phenomenon that occurs in many different tissues. A particular example is that cells with damaged DNA are induced to

undergo apoptosis by a mechanism involving p53, the so-called guardian of the genome. In the context of oral tolerance, it has been shown that activation precedes apoptosis as shown by increased levels of cell marker expression and cytokine production. CD44 is increased and CD45RB is reduced on these cell surfaces prior to apoptosis, both of which are a sign of cellular activation. In addition IFN-γ, IL-4 and IL-10 levels are increased before apoptosis suggesting that both T_H1 and T_H2 cells are activated. With more prolonged feeding all these cytokines fall to zero, indicating that apoptosis occurs in both cell types. This suggests high dose feeding initially activates these immune cells, but further signalling (or possibly lack of signalling) causes these cells to undergo apoptosis.

The experiments described above use transgenic strains of animal that are fed antigen at supra-physiological doses. Furthermore clonal deletion was not observed in a different transgenic mouse model with myelin basic protein TCR[150]. It is unclear whether the results from such models can be generalised to non-transgenic models that ingest physiological quantities of antigen. It has proved difficult to detect clonal deletion in non-transgenic models due to the small proportion of T cells that are specific for the fed antigen. Recently, attempts have been made to overcome this problem by transferring a limited number of cells from transgenic animals into wild-type animals. Clonal deletion has not been reported in these models[147;151]. Thus further work is necessary to confirm that clonal deletion can mediate oral tolerance when antigen is encountered by normal animals in physiological doses.

2.7 Effect Of Dose On The Type Of Oral Tolerance Induced

The above review illustrates that in different animal models, different mechanisms of inducing tolerance have been discovered. Which factors influence the development of tolerance down one pathway or another? The most important differences between these studies relate to the dose and duration of antigen feeding. For example, in Whitacre's

experiments on abrogating EAE, which provided evidence for clonal anergy, there were several differences from the experiments which described active suppression. These include the dose of antigen (5mg/feed v. 0.5mg/feed), the fact that it was administered with a protease inhibitor and the fact that the mice were fed on an empty stomach. All these factors have the effect of increasing the dose of intact protein that is absorbed by the intestinal mucosa. This has lead to the theory that low dose feeding results in the production of active suppression or immune deviation, whereas high dose feeding results in the production of clonal anergy or clonal deletion[152].

One can review some of the early studies on oral tolerance and reinterpret them in the light of this hypothesis. For example, it explains the differing responses of the systemic humoral response that were discussed above. Those experiments that showed a rise or priming of the humoral response may have been observing a T_H1 to T_H2 switch caused by low dose feeding. On the other hand those studies that showed a fall in humoral responses may have been observing clonal anergy caused by higher doses of feeding. The results of Peng *et al*[16] show this split in one study.

These observations have lead to the development of experimental models looking at the different causes of tolerance related to differences in the dose and length of feeding. One study worth mentioning in detail is that of Gregerson *et al* who fed mice high and low dose of S-ag and measured the effect on disease activity of subsequently induced experimental autoimmune uveoretinitis (EAU)[153]. By feeding peptide fragments, they were able to demonstrate that low dose feeding (250μg/feed) offered protection when EAU was induced with the whole S-ag or even when a different immunogenic epitope from the S-ag was used. This finding is likely to represent bystander suppression. In addition, they were able efficiently to transfer resistance to non-fed animals. Conversely high dose feeding (5mg/feed) offered protection if the disease was induced with the same epitope as the feed, but not if a different epitope from the same antigen was used. Further, transfer of resistance

to EAU was much less effective when cells were taken from high dose animals. These results suggest that low dose feeding results in active suppression, but high dose feeding results in anergy. Logically it would seem that the anergic process must affect either the suppressor cells or the T_H cells that provide the necessary "help" to induce them, since transferring T cells does not result in transfer of tolerance in these systems. Other authors have confirmed these observations by showing that low dose feeding causes active suppression, but that high dose feeding causes clonal anergy in an experimental system using myelin basis protein[134].

2.7.1 Mechanisms of Tolerance Combine to Produce Oral Tolerance

Thus it seems that the mechanism of tolerance induced varies depending on the dose of antigen fed. It seems that in different animal models, clonal deviation, active suppression, clonal anergy and clonal deletion can all induce tolerance. It seems likely that these mechanisms can all occur in one species and combine to cause tolerance to most antigens in most doses encountered. Furthermore, these mechanisms overlap to ensure that tolerance occurs, as discussed below.

Lowest doses of feeding seem to cause tolerance by clonal deviation, with the production of a predominantly humoral T_H2 response[122]. Also at low doses active suppressor cells are formed[135]. T_H2 cells themselves may act as suppressor cells, particularly of DTH[80]. In addition, once a T_H2 response is produced, it can be self-perpetuating. Naïve T cells will be induced to differentiate into T_H2 cells by the T_H2 cytokines produced locally. As has been shown, IL-4 and IL-10 will cause T_H1 cytokine genes to be switched off, thus causing the naïve cells to differentiate into T_H2 cells[68;70]. More specific suppressor cells, possibly producing high levels of TGF-β, are also produced[133]. TGF-β producing cells may be able to inhibit both cell-mediated and humoral immune responses[131;132].

With more prolonged feeding anergy becomes a more important mechanism to induce and maintain tolerance. Initially T_H1 cells are more susceptible to anergy than T_H2 cells[149;154]. Thus at this stage, anergy will also help to maintain the balance of the immune response to the oral antigen towards the antibody producing, T_H2 type response. However with further feeding, T_H2 cells also become anergic[130] and both limbs of the immune response may become suppressed.

It is interesting that there is evidence that anergic cells can secrete some cytokines, including TGF-β and IL-4[155]. Therefore there may be a stage where anergic cells can secrete suppressive cytokines, thus providing another area of overlap between active mechanisms of tolerance production and clonal anergy in the maintenance of oral tolerance[134]. Other authors have suggested an alternative mechanism whereby anergic cells can act as suppressor cells. Using an *in vitro* assay, these authors showed that human anergic T cells could inhibit antigen specific and allospecific T cell proliferation. The authors felt that their experiments suggested that the anergic cells were suppressive in a passive manner by competing for IL-2 and antigen ligands[156]. Another report suggests that T cells that are rendered anergic by immobilised anti-CD3 monoclonal antibody could transfer tolerance. In this report suppressive cytokines were not secreted by these anergic cells and neutralising antibodies to IL-4, IL-10 or TGF-β did not prevent transfer of tolerance. Furthermore cell-to-cell contact appeared to be required for transfer of tolerance[157]. It should be noted that in these experiments, the anergic cells were not obtained by inducing oral tolerance, and it was an *in vitro* system. Therefore these results may not be applicable to *in vivo* oral tolerance. However, experiments in a mouse model have shown that CD4+ cells rendered anergic by high dose feeding could transfer tolerance to SCID or nude mice[116]. Thus anergic cells may also act as immunoregulatory cells to control the immune response.

With further feeding clonal anergy[141] and possibly clonal deletion[133] may become the overwhelming mechanism of tolerance production and all aspects of the immune response are suppressed.

2.7.2 Survival Advantages for This Model of Oral Tolerance

If one considers the type of antigen that is likely to be encountered at a given dose, it seems that the system outlined above would be of benefit to the host animal. Pathogenic bacteria and viruses are likely to be encountered for a short time, and possibly at a relatively high dose. These are not conditions that induce tolerance readily, and are likely to induce a T_H2 response. The advantage of a good humoral response is that it will neutralise any toxins or pathogenic binding sites thus keeping these potentially harmful organisms in the lumen, while avoiding a cell mediated immune response with potential attendant tissue damage. Food antigens are encountered over prolonged periods of time and in relatively high doses. These conditions are ideal for tolerance formation, and are likely to produce clonal anergy. Therefore, there will be reduced humoral immune response to food antigens. This has advantages to the host in that these antigens are harmless and therefore any immune response to them is unnecessary, and indeed wasteful.

Thus this model, with two or more mechanisms of tolerance production that are mutually compatible and overlapping, provides an explanation which fits with the majority of the published data. In addition it provides theoretical advantages to the host based on the type of antigens that are likely to be encountered at the relevant doses.

2.8 Evidence for Oral Tolerance in Humans

The vast majority of the above work has been published in animal models. There is very little direct work looking at oral tolerance in man. There is one study investigating the immunological effects of oral antigen exposure directly[9]. This study used KLH, which is a neoantigen. A control group was given a sub-cutaneous injection schedule of KLH. The

active group was fed two 5-day courses of KLH over 3 weeks at a dose of 50mg per day. The immune response was assessed by measuring DTH, *in vitro* lymphocyte proliferation and humoral responses. They found that cell-mediated immune responses were suppressed, whereas humoral responses were primed. They concluded that oral tolerance did occur in humans, but made no comment on the mechanisms involved. It is possible however to suggest that the induction of immunomodulatory cells (?T_H2 cells) would explain their observations.

This is the only completed, controlled study of oral tolerance reported in the literature, although a pilot study has also been published[158]. There are other studies that imply the existence of oral tolerance in humans, included that reported by Lowney[159;160]. He fed volunteers a contact sensitisor (dinitrochlorobenzene) before attempting to sensitise them to this antigen. He found that provided an adequate oral dose was given (greater than 20mg), there was a reduction in the degree of sensitivity. These experiments clearly suggest that oral tolerance has developed in the T cell compartment in response to low dose feeding.

Another study that implies the existence of oral tolerance in man is that of Korenblat *et al*[19]. This group was investigating humoral responses to food antigens. It is well known that there are antibody responses to food antigens in healthy people. Korenblat *et al* attempted to provoke these responses by feeding people high doses of antigen or by giving intra-dermal immunisation with the food antigen. They found that there was no measurable DTH response to any of their injections. The humoral responses fell into two categories. One group had low levels of antibody to start with and showed no rise in antibody levels following further exposure to antigen. The second group had higher levels of baseline antibodies, and this group did show a small rise in antibody levels following systemic exposure. These findings were reported 30 years ago and the authors concluded that the gastrointestinal mucosa processed antigen in a different way than the systemic immune response. It is possible in the light of subsequent work to suggest that this difference in processing may be due to the production of an initial T_H2 response - hence producing the detectable levels of antibody

seen. With further exposure, other mechanisms of tolerance production such as clonal anergy may be induced. If the anergy affects T_H2 cells, then there would be no help provided for the humoral response thus explaining the failure of the antibody response to increase following immunisation.

These hypotheses are supported by the fact that antibody levels to food antigens fall with age[161]. One can suggest that more prolonged exposure to antigen that occurs with increasing age results in the production of more powerful tolerance producing mechanisms, which inhibits further antibody production to food antigens. Prolonged exposure would then suppress this response. Further indirect evidence that oral tolerance occurs in humans can be derived from the observation that secretory antibodies to food antigens are rare[12].

It thus seems clear that oral tolerance can occur in humans. The only paper that looked at the immune responses in the laboratory in a controlled study was that of Husby and colleagues[9]. Their results could be explained by the production of an immunomodulatory cell response following encounter with low dose antigen. The studies looking at dietary antigens (e.g. those of Scott *et al*[161] and Korenblat *et al*[19]) imply a more profound degree of tolerance, since the humoral immune responses are suppressed as well. These results infer that the dose of antigen fed influences the mechanism of tolerance production, in the same way that has been suggested in animal models (see above). My studies were designed to investigate further the ability of different feeding schedules to induce tolerance in the different compartments of the immune system.

2.9 Clinical Implications of Oral Tolerance

2.9.1 Treatment of Autoimmune Diseases

One area where oral tolerance may be of clinical benefit is the treatment of autoimmune diseases. By feeding the self-antigen towards which the autoimmune process is directed, one can in theory down-regulate the immune response and thus abrogate the effects of the

disease. Oral tolerance is likely to be safe, cheap, free of side-effects and antigen specific compared to the current treatment of immunosuppressant drugs[162].

Many animal models of autoimmune disease have been treated by feeding the oral antigen towards which the autoimmune response is directed, and shown that the effects of the disease can be reduced. These include feeding insulin to NOD mice[163], giving myelin basic protein to animals with EAE, a model of multiple sclerosis[115;141], feeding collagen to mice with adjuvant arthritis, a model of rheumatoid arthritis[30], and using protein S antigen to inhibit EAU[153]. Finally transplant rejection, which is not an autoimmune disease, but is an undesired immune response to foreign tissue, can be reduced by feeding antigens from the transplanted organ in the peri-transplant period[164;165].

These experiments have been so encouraging that trials of therapy for human autoimmune disease using oral antigens to induce tolerance have been undertaken. Two preliminary studies have been published; the first feeding patient with rheumatoid arthritis type II collagen[166], and the second feeding patients with multiple sclerosis myelin antigens[167]. Both these trials were preliminary attempts to prove safety, and neither reported any adverse events. On the other hand, neither trial was large enough to be able to draw conclusions on efficacy. More recently, larger trials have been published with generally disappointing results. Most work has been performed on patients with rheumatoid arthritis. One study randomised 90 patients with early rheumatoid arthritis to receive oral bovine type II collagen or placebo, but found no statistically significant differences between the two groups. The authors did claim that a minority of patients had a good response and felt that further work was justified[168]. Other authors investigated a group of 190 patients with active rheumatoid arthritis to receive 6 months of oral bovine type II collagen or placebo. No statistically significant results were found[169]. Finally, another group investigated whether oral feeding of chicken type II collagen would enable the dose of immunosuppressants to be reduced in patients with stable long-standing rheumatoid arthritis that was controlled on methotrexate. They found that the group that received oral collagen and discontinued methotrexate had a

significant deterioration of disease activity compared to those maintained on methotrexate[170]. Despite these disappointing results, one trial has reported a positive benefit of feeding type II chicken collagen. 274 patients with active rheumatoid arthritis were randomised to receive collagen or placebo and reported that, on one measure of disease severity, there was a significant improvement in patients that received the lowest oral dose of collagen tested[171]. Interestingly, this group observed that the lowest dose of oral collagen they administered, which was lower than those used in other trials, gave the best results. It is possible that this dose was most effective at inducing regulatory cells that could modify disease activity.

There is a report of the use of oral retinal S antigen and/or a mixture of soluble retinal antigens in the treatment of immunosuppressant dependent uveitis. No significant difference was noted between any of the treatment groups compared to placebo, although there was a trend towards a more successful tapering of immunosuppressants in the group fed S antigen alone[172].

In many human autoimmune diseases, the exact antigen responsible for the disease is unknown, and it is likely that more that one antigen is responsible in most cases[173]. Further, even if an autoimmune immune disease is initially directed against one antigen, animal models suggest that there is spread of autoimmunity, both to other epitopes on the same antigen[174] and to different antigens in the same target organ[175]. Therefore feeding a solitary antigen may not be able to suppress the whole autoimmune response, particularly if it is not known if the fed antigen is involved in the autoimmune response. This is particularly true if clonal anergy or clonal deletion is the mechanism responsible for the tolerance, since these mechanisms are exquisitely antigen specific for the fed antigen.

The theoretical exception to this rule is the induction an active suppressor cell which may cause bystander suppression in the organ affected by the disease. At present the understanding of the mechanisms of oral tolerance in the human is poor. Improving our knowledge of how oral tolerance occurs in humans may lead to techniques to improve treatment of autoimmune disease using oral tolerance.

2.9.2 Diseases of Gastrointestinal Inflammation

There are several diseases of the gastrointestinal tract that are caused by inflammation in the mucosa of the bowel. Clearly, inflammation to harmless antigens should not occur if oral tolerance is operating normally. It may therefore be instructive to consider these diseases in the terms of oral tolerance since this may suggest new theories of pathogenesis. There are two diseases of gastrointestinal inflammation that will be mentioned here.

2.9.2.1 Coeliac Disease

Coeliac disease is caused by an abnormal immune response to the gliadin fragment of gluten which is a protein found in many cereals. There is a detectable cell-mediated[176;177] and humoral immune response[178] to gluten in these patients. Interestingly there is also a detectable humoral immune response to other antigens including anti-endomysial antibodies and anti-reticulin antibodies[178], which may suggest spread of the immune process between antigens.

Recently the target of anti-endomysial antibodies has been identified as tissue transglutaminase (tTG), which is a self-antigen[179]. The authors suggest that gliadin may be able to bind to tTG and that the combination is morphologically altered to produce a neoantigen. The immune response may be initiated against this neoantigen, and then be propagated by antigenic spread. If this theory were indeed true, this would represent an unusual and interesting way in which the protective effects of oral tolerance may be overcome.

2.9.2.2 Inflammatory Bowel Disease

IBD is usually thought of as two similar but distinct diseases; namely ulcerative colitis (UC) and Crohn's disease (CD). UC affects the colon alone and only involves the superficial layers. It is suggested that it may be mediated by a T_H2 response. CD can affect the whole of

the bowel, both in length and in wall thickness. The immune responses seem to be due to a predominantly T_H1 response. In each case the antigen towards which the immune response is directed is unknown, although many candidate antigens have been suggested. These include lumenal bacteria such as bacteroides, measles virus, paratuberculosis infections and fluoride.

In this condition, one can postulate that the disease is not caused by an immune reaction to a single antigen, but by a defect in oral tolerance resulting in abnormal immune reactions to many antigens. There is some evidence that this may occur, since lamina propria mononuclear cells from patients with IBD reacted to bacterial sonicates of intestinal flora, whereas control patients and those with non-active disease did not[27]. This suggests that tolerance to intestinal flora may have been broken. Further there is evidence that T cells isolated from gut tissue of patients with CD expressed a T_H1 pattern of cytokine secretion. Control patients and those with UC did not secrete a typical T_H1 pattern of cytokines[180]. It is possible that CD is caused by an early defect in oral tolerance, since it appears to be mediated by a T_H1 response which is abrogated early in the course of antigen feeding in normal subjects. UC, mediated by a T_H2 response, may be due to a later defect in oral tolerance such as a failure of anergy in the T_H2 compartment.

These statements are purely hypothetical, but ideas such as these do provide us, as gastroenterologists, with an incentive to improve understanding of the mechanisms of tolerance in humans. The methods devised in this thesis to study tolerance could be applied to patients with IBD to assess whether oral tolerance is defective in these patients.

2.10 Aims of this Thesis

The literature on oral tolerance in animals suggests that the gut immune system can respond to oral antigen by producing tolerance by several different mechanisms. Differences in the type of antigen, the animal model and particularly the dose and length of feeding of antigen alter the type of tolerance produced. The mechanisms do however appear to overlap and

therefore ensure that an appropriate type of tolerance is produced in response to a wide variety of antigens in a wide range of doses.

There is very little work looking directly at the mechanisms of tolerance production to different antigens and feeding schedules in humans. The aims of this thesis were therefore to develop a protocol to demonstrate the presence of oral tolerance in humans. Assays to measure different aspects of the immune response were developed, including measures of the humoral and cell-mediated immune responses. Using these assays, I aimed to define the immunological consequences of oral antigen exposure. Furthermore, I hoped be able to draw conclusions on the mechanisms of tolerance production in humans by comparing the immune responses observed in humans to those seen in established animal models.

3 MATERIALS AND METHODS

3.1 Introduction to Experimental Protocol Used to Demonstrate Oral Tolerance

The protocol that I used to demonstrate oral tolerance was based on that used by Husby and colleagues in their report of oral tolerance to a neoantigen in humans[9]. A control group received an immunisation schedule of two subcutaneous injections of KLH. The immune response was characterised by assaying for delayed-type hypersensitivity (DTH), anti-KLH antibody production and *in vitro* lymphocyte proliferation to KLH. The test group were fed a course of KLH before receiving the same immunisation schedule as the control group. The same measures of the immune response to KLH were made, and any differences between the two groups were attributed to oral tolerance. The timing and dose of immunisations are shown in table 3.1.

The immune response to a different antigen, OVA, was also measured. OVA is the major constituent of egg white and is present in the normal British diet. Therefore, this group was assumed to have previously ingested substantial quantities of OVA over a prolonged period and no additional feeding was given. This group was given two sub-cutaneous injections of OVA in a similar immunisation schedule to that used in the KLH experiments. DTH responses, anti-OVA antibody responses and *in vitro* lymphocyte proliferation responses to OVA were measured. The immune responses to OVA were compared with those obtained in the KLH experiments.

Table 3.1 – Protocol for the induction and demonstration of oral tolerance. This table shows the dose and timing of feeding and injection schedule for the induction and demonstration of oral tolerance used in this thesis. Also shown are the timings at which samples were taken for measurement of anti-KLH antibody responses, lymphocyte proliferation responses and DTH responses.

Day	Control group	KLH-fed group 1	KLH-fed group 2	KLH-fed group 3	Assays performed
1	No treatment	Fed KLH 10mg/day for days 1-10	Fed KLH 50mg/day for days 1-10	Fed KLH 50mg/day for days 1-10 and 14-19	Antibody responses Lymphocyte proliferation
21	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Antibody responses Lymphocyte proliferation
31	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Sub-cutaneous KLH injection (200μg)	Antibody responses
42	Intra-dermal KLH injection (10μg)	Intra-dermal KLH injection (10μg)	Intra-dermal KLH injection (10μg)	Intra-dermal KLH injection (10μg)	Antibody responses Lymphocyte proliferation DTH responses
43 and 44					Skin tests read for erythema and induration

3.2 Preparation of Antigens for Immunisation

KLH and OVA were both obtained from Sigma-Aldrich Co and were manufactured to American Food and Drug Administration standards. They were therefore of a sufficient standard of purity to be used for immunisation in humans. The injections were prepared in the aseptic unit of the Western General Hospital. The antigens were dissolved in 0.9% weight (wt) / volume (vol) sterile saline and the solution was diluted to the required concentration of antigen. The solution was passed through a 0.45 micrometer (μm) filter (Millipore) to remove any large insoluble particles, including most bacteria. The required volume was then drawn up in a sterile syringe and dispensed for use.

All injections were given using a sterile needle and syringe. The immunising injections were given into the triceps fat pad.

3.3 Inclusion and Exclusion Criteria for Subjects Recruited

Healthy volunteers age 15-50 years were recruited. There were two principal exclusion criteria. Firstly, the subjects had to have no history of allergy to seafood if they were being included in the KLH arm of the study or to eggs if they were entering the OVA arm of the study. Secondly, women of childbearing age were excluded if they were pregnant or lactating, and had to be using contraception for the course of the study. Informed written consent was obtained.

3.4 Delayed-Type Hypersensitivity (DTH) Testing

DTH testing was performed by injecting a 10 micrograms (μg) of antigen in 10 microlitres (μl) of sterile saline intra-dermally into the volar aspect of the forearm. Two measures of DTH were made. Firstly, the DTH response was graded as positive or negative depending on whether any induration of the skin was detectable at the site of the injection. Secondly, the mean diameter of erythema was calculated by averaging the maximum diameter of erythema

and the diameter of erythema taken at 90^0 to the maximum diameter. The measurements of DTH were taken at 24 and 48 hours. All measurements of DTH response were made by the same observer to eliminate any inter-observer bias. The observer was not blinded as to which groups the volunteers belonged.

3.5 Measurement of Antibody Responses

3.5.1 Introduction to the Principles of ELISA

To detect antigen-specific antibody of different isotypes, a five-stage enzyme linked immunosorbent assay (ELISA) was employed. Briefly, a plastic 96 well microplate was coated with the relevant protein antigen. This was followed by incubation with a blocking solution to prevent any subsequent non-specific binding of the antibody to be assayed. Next, the serum samples under test were added to duplicate wells. Any antigen specific antibody would bind to the antigen-coated wells. At the fourth stage, an anti-isotype antibody conjugated to an enzyme was added. Finally, the chromogenic substrate of the enzyme was added to each well. The absorbance of light at the appropriate wavelength was directly proportional to the amount of antigen-specific antibody of the isotype under test present in the sample.

3.5.2 Serum Collection and Storage Prior to Use in ELISA

20 millilitre (ml) of peripheral blood was collected by venepuncture into a plain blood tube and allowed to clot for at least 10 minutes at room temperature. The tube was centrifuged at 1400 gravity (g) for 10 minutes. The serum was collected and stored at -70^0C in 200μl aliquots until use.

3.5.3 Anti-OVA IgG and IgA ELISA

3.5.3.1 Anti-OVA IgG and IgA ELISA Methods

This is a well-established assay in the GI laboratory and, therefore, the conditions for the assay had already been developed and optimised. OVA was dissolved in 0.05M carbonate-bicarbonate buffer pH 9.6 at a concentration of 5μg per ml. 100μl of this solution was added to each well of an Immunlon® 2, 96 well flat bottomed microplate and incubated at 22-24^{0}C for 5 hours. The plate was then washed 3 times with 0.9% wt/vol saline – 0.05% vol/vol Tween-20 solution, using an automatic plate washer.

Next 100μl of assay diluent (0.9% saline, 1% wt/vol BSA and 0.05% Tween-20 solution) were added to each well as a blocking solution. The plates were incubated for 2 hours at 22-24^{0}C. The plates were washed as before.

Serum samples were diluted in assay diluent before use in the assay. For IgA anti-OVA antibody analysis, samples were diluted 1 in 50, and for IgG anti-OVA antibody analysis, the dilution was 1:500. 100μl of each serum sample was added to duplicate wells. The plates were incubated for 16 hours (overnight) at 4^{0}C.

After washing as before, 100μl of alkaline phosphatase conjugated anti-human IgG or IgA goat antibody (Sigma) was added to appropriate wells. Both antibodies were diluted 1:500 with assay diluent before use. The plates were incubated for a further 5 hours at 22-24^{0}C.

After a final wash, 100μl of the chromogenic substrate p-nitrophenylphosphate (PNPP) at a concentration of 1mg/ml in diethanolamine (DEA) substrate buffer (10% vol/vol DEA, 0.0005M $MgCl_2.6H_2O$, 0.02% $NaNH_3$) pH 9.8 was added to each well. The plate was then placed in an ELISA reader that monitors the plate until the top standard reached an optical density (OD) of 1 at 405nm, at which time the ODs in all the wells were read at the same wavelength of light.

3.5.3.2 Calculation of Values of Anti-OVA Antibody

The GI laboratory has developed known high standards of anti-OVA IgG and IgA by pooling serum from patients with high titres of these antibodies.

For the IgA anti-OVA assay, the standard was diluted 1 in 265 in assay diluent to give a top standard of 1600 experimental units (U). Further doubling dilutions were performed down each plate to a bottom standard of 25U. For the IgG anti-OVA assay, after an initial 1 in 675 dilution to give a top standard of 1600U, the standard was doubly diluted down each plate to a bottom standard of 25U. A standard curve was fitted to these points using a quadratic equation. The value for each test sample was derived from the standard curve. Thus the titre of antibody (in units) in each sample was calculated by comparing the values to those of sample with a known high titre of antibody that was arbitarily given a value of 1600 units.

3.5.4 Anti-KLH IgG and IgA ELISA

3.5.4.1 Anti-KLH IgG and IgA ELISA Methods

KLH was dissolved in 0.05M carbonate-bicarbonate buffer pH 9.6 to a concentration of 1μg/ml. 100μl of this solution was added to each well of an Immunlon® microplate. The plate was then incubated overnight (16 hours) at 4^0C and then washed 3 times with wash buffer (0.9% wt/vol saline, 0.05% vol/vol Tween-20 solution) using an automatic plate washer.

100μl of a blocking solution (5% vol/vol foetal calf serum (FCS) in phosphate buffered saline (PBS) 0.01M, pH 7.4) was added to each well, and the plate was incubated for 6-8 hours at 22-24°C. The plate was washed 3 times as before.

Serum samples were diluted 1 in 200 with assay diluent (1% vol/vol FCS, O.05% Tween-20 in PBS). 100μl of each sample was added to duplicate wells and the plate was incubated overnight (16 hours) at 4^0C. The plate was again washed 3 times as before.

Anti-human IgA and IgG-specific, alkaline phosphatase conjugated goat antibodies (Sigma) were diluted 1:1000 and 1:2000 respectively with assay diluent prior to use. 100μl of the appropriate antibody conjugate was added to each well and incubated 22-24°C for 5 hours. Following a final wash, 100μl of 1mg/ml PNPP in DEA substrate buffer was added to each well. The plate was then placed in an ELISA reader that monitors the plate until the top standard reached an OD of 1 at 405nm, when the ODs of all test wells were read at the same wavelength of light.

3.5.4.2 Calculation of Values of Anti-KLH Antibody

One volunteer received a secondary immunisation with KLH seven weeks after she had received the standard immunisation regime. Serum was collected, as described above, 10 days after this injection and aliquots were stored at -70^{0}C. This sample contained high levels of anti-KLH IgG and IgA and was used as the high standard in each experiment.

The standard was diluted 1:200 with assay diluent to give a top standard of 1600U. Doubling dilutions of this standard were performed down each plate, to give a bottom standard of 25. A standard curve was fitted to these points using linear regression for anti-KLH IgG antibodies and a quadratic equation for anti-KLH IgA antibodies. The value of each test samples was derived from this standard curve.

3.5.5 ELISA Methodology for Measuring IgG1 and IgG2 Anti-KLH Antibodies

The protocol used to measure anti-KLH IgG was adapted to allow measurement of IgG1 and IgG2 subclasses of anti-KLH IgG. Alkaline phosphatase labelled anti-human IgG1 and IgG2 antibodies (Southern Biotechnologies Associates Inc) were diluted 1:1000 with assay diluent, and these solutions were used at the fourth step in the assay. A standard curve was obtained using doubling dilutions of the high standard obtained as previously described.

3.6 Lymphocyte Proliferation Assay

3.6.1 Mononuclear Cell Isolation

Peripheral blood was collected by venepuncture into a lithium heparin tube. The blood was diluted 1:3 to 1:4 with normal (0.9% wt/vol) saline. 15ml of diluted blood was carefully layered over 5ml of Histopaque 1077 (Sigma) in a sterile universal container and centrifuged at 500g for 25 minutes. The mononuclear cells, which lie at the Histopaque/plasma interface, were collected with a sterile pipette and placed in a fresh sterile universal container.

The cells were washed twice in RPMI 1640 medium (Sigma) and recovered by centrifugation at 500g for 15 and then 12 minutes. The cells were resuspended in 3ml of culture medium (100ml of RPMI 1640, 10ml of FCS, 2ml of 200 millimolar (mM) L-glutamine, and 1ml of penicillin-streptomycin solution containing 10,000 units of penicillin and 10μg of streptomycin per ml (all Sigma)). The number of mononuclear cells was counted and the concentration of cells was adjusted to $3x10^6$ cells per ml of culture medium.

3.6.2 *In vitro* lymphocyte proliferation assay

Antigen solutions were prepared by dissolving antigen in culture medium. KLH was prepared to a concentration of 10μg per ml and OVA to concentrations of 10 and 100μg/ml. 100μl of cell suspension were placed in wells of a 96 well flat-bottomed plate. 100μl of appropriate antigen solution were added to triplicate wells. 100μl of culture medium alone were added to 3 wells as a negative control, and 100μl concanavalin A (con A), dissolved in culture medium in a concentration of 6.6μg per ml, were added to 3 wells as a positive control. The plates were cultured for 98 hours at 37^0C in oxygen and 5% CO_2. For the final 6 hours of culture, 20μl of ^{3}H-thymidine, containing a radiation dose of 50μCu, were added to each well.

The contents of each well were aspirated onto Filtermat A filter paper (Wallac), which was dried for 2 minutes at medium power in a 550 Watt microwave oven. Multiplex scintillation paper (Wallac) was placed on the filter paper and melted using a hot plate at 90^0C. A betamax scintillation counter was used to quantify the amount of ^{3}H-thymidine incorporated into the cells in each well. The scintillation from each well was counted for 5 minutes

The proliferation index for each set of triplicate wells was calculated by dividing the mean scintillation from stimulated cells by the mean scintillation of the negative control. Thus if there was no antigen specific stimulation, the proliferation index would have a value of one.

3.6.3 Use of Dynabead-Antigen Complexes

In certain experiments, OVA was coated onto Dynabeads M-280 tosylactivated (Dynal UK Ltd) prior to use in the lymphocyte proliferation assay. Dynabeads are uniform, superparamagnetic beads coated with super-reactive tosyl groups. The super-reactive tosyl groups will react with primary amino acid groups on proteins, and thus the beads can be coated with protein antigen. The magnetic properties of the beads allow them to be easily manipulated experimentally. The diameter of the beads is 2.8μm, and thus they should be phagocytosed by APCs.

3.6.3.1 Preparation of Dynabead-Antigen Complexes

The Dynabeads were supplied in an aqueous suspension of 6 x 10^8 beads/ml. The required quantity of beads was washed prior to coating as follows. The beads were resuspended, transferred to an Eppendorf tube and placed in a magnet to remove the beads from suspension. The supernatant was discarded and the beads were resuspended in 1ml of 0.1M sodium phosphate buffer pH 7.4 (2.62g $NaH_2PO_4xH_2O$ and 14.42g $Na_2HPO_4xH_2O$ in 1000ml of distilled water) by mixing for two minutes. The tube was again placed in the

magnet and the supernatant discarded. The beads were resuspended in sodium phosphate buffer at a concentration of $6x10^7$ beads/ml.

To coat the beads with OVA, 500µl of OVA at 60µl/ml in sodium-phosphate buffer were added to 500µl of the bead suspension and mixed overnight at 37^0C. During this time, the manufacturer reports that 30-80% of protein should bind to the beads via the tosyl group. The beads were then washed twice in PBS at pH 7.4 with 0.1% wt/vol BSA. A further wash was performed using 0.2 molar (M) Tris at pH 8.5 with 0.1% wt/vol BSA. The Tris should bind to any remaining tosyl groups. A final wash with PBS at pH 7.4, 0.1% BSA removed excess Tris. The beads were stored in PBS, 0.1% wt/vol BSA and 0.02% wt/vol sodium azide at 4^0C until use.

A further quantity of beads was treated exactly as described above but without OVA dissolved in the coating solution. This sample of beads was used as a control to ensure that any differences in proliferation were due to OVA and not due to the beads themselves.

3.6.3.2 Use of Complexes in Lymphocyte Proliferation Assay

Prior to use in the lymphocyte proliferation assay, the beads were washed in PBS to remove the sodium azide. The beads were resuspended in culture medium at a concentration of 7 x 10^5 beads per ml giving a concentration of 10µg of OVA coated beads/ml. 100µl of bead suspension was added to 100µl of cell suspension containing 3 x 10^6 cells/ml in triplicate wells of a 96 well flat bottom plate. The proliferation assay was performed as described above. The proliferation index was calculated by dividing the mean scintillation count in the wells stimulated with OVA-bead complex by that in the wells stimulated with the beads prepared without OVA.

3.6.4 Use of Lymphocyte Proliferation Assay to Investigate Bystander Suppression

Mononuclear cells were prepared as described above at a concentration of $3x10^6$ cells/ml. 100,000/ml tuberculin PPD (Health Authority) was diluted 1/100 with culture medium. KLH

(or OVA as appropriate) was dissolved in culture medium at a concentration of 10μg/ml. Solution of KLH (or OVA) at 10μg/ml and PPD 1000 unit/ml in culture medium were prepared.

100μl of each antigen solution were added to triplicate wells of a 96 well flat-bottomed plate. 100μl of the cell suspension was added, and the assay was performed as previously described.

PPD is used to inoculate against TB, and therefore will cause a positive proliferation response in the cells from most people[181]. A suppression index was calculated by dividing the mean proliferation of cells cultured with PPD by the mean proliferation of cells cultured with PPD and KLH (or OVA) together. If there was no detectable bystander suppression, the suppression index would be one, and if there was bystander suppression, values of greater than one would be recorded.

3.6.5 Reversal of Anergy with IL-2

Mononuclear cells were prepared as described above at $3x10^6$ cells per ml in culture medium. 1.5 ml of cell suspension was cultured with 75 international units of recombinant human IL-2 (Sigma) in a 6 well flat-bottomed culture dish for 92 hours at 37^0C with 95% O_2 and 5% CO_2. The cells were transferred to a sterile universal. The flat-bottomed well was washed with RPMI 1640 medium and scraped with a cell scraper to ensure all possible cells were transferred. The cells were washed twice in RPMI 1640 medium, recovered by centrifugation at 500g for 10 minutes and finally resuspended in 1.5 ml of culture medium.

OVA was dissolved in culture medium at concentrations of 10μg/ml and 100μg/ml. 100μl of cell suspension and 100μl of OVA solution were added to triplicate wells of a 96 well-flat bottomed plate. 100μl of culture medium and 100μl of conA at a concentration 6.6μg/ml were added to cells in triplicate wells to act as a negative and positive control. The plates

were incubated for a further 98 hours at 37^0C with 95% O_2 and 5% CO_2, and lymphocyte proliferation was measured by ^{3}H-thymidine incorporation as previously described.

APCs are required for the lymphocyte proliferation assay to work. APCs are adherent and may stick to plastic wells during the first incubation and therefore may not be transferred to the wells used for the second stage of the assay. Three different strategies were used to attempt to overcome this problem. Firstly, a cell scraper was used after the first incubation to attempt to remove any adherent cells from the plastic and ensure that they were contained in the cell suspension for the second phase of the experiment. Secondly, $3x10^5$ mononuclear cells in 100µl of culture medium were added to wells in a 96-well plate and incubated for 92 hours at 37^0C while other cells were being incubated with IL-2 as described above. After this incubation, the supernatant was removed and the wells (which I postulated would now contain adherent APCs) were used for the second stage of the experiment as described above. Lastly, volunteers were venesected again at day 4 and fresh mononuclear cells were isolated as previously described. 100µl of culture medium containing $3x10^5$ cells were added to the IL-2 pre-cultured cells and antigen to act as APCs for the second incubation.

3.7 Cytokine Profile Estimation

Animal models suggest that some mechanisms of oral tolerance are associated with the activation of specific T cell subsets, which can be identified by the cytokine profile that they secrete. I attempted to measure cytokine levels secreted by activated mononuclear cells in the lymphocyte proliferation assay by the following methods.

3.7.1 ELISA Measurement of Cytokine Levels in Cell-Culture Supernatant

3.7.1.1 Collection of Samples

100μl of culture medium containing $3x10^5$ mononuclear cells were incubated with 100μl of 10μg/ml KLH in culture medium for 90 hours at 37^0C in 95% O_2 and 5% CO_2. The supernatant was collected from separate wells after 24 and 90 hours of culture and stored at – 70^0C until use.

3.7.1.2 ELISA Measurement of Cytokines

Commercial ELISA kits (R&D Systems) were used to measure the levels of IL-4 and IFN-γ in cell culture supernatants. The ELISA was run according to the manufacturer's instructions. Briefly, a plate pre-coated with anti-cytokine monoclonal antibody was supplied by the manufacturer. Each cell culture supernatant was diluted 1/10 with the assay diluent supplied. 200μl of each test sample was added to each well and the plates were incubated for 2 hours (IL-4 assay) or 2.5 hours (IFN-γ assay) at room temperature. After washing the plate with the wash buffer supplied, 200μl of anti-cytokine, hydrogen peroxidase conjugated monoclonal antibody was added to each well. The plates were incubated for 2 hours at room temperature and washed. 200μl of a substrate solution containing hydrogen peroxidase and tetramethylbenzidine was added to each well. After a 20-minute incubation at room temperature, 50μl of 2N sulphuric acid was added to each well. The OD of each solution at 450 nanometers (nm) was recorded by an automated ELISA reader.

IL-4 and IFN-γ standards were provided with each kit. Doubling dilutions of the standard were performed on each plate, from which a standard curve was generated. The amount of cytokine in the cell culture supernatants could then be calculated.

3.7.2 mRNA Expression by Lymphocytes

3.7.2.1 Extraction of RNA from Mononuclear Cell Cultures

mRNA is a very delicate structure, subject to degradation by RNases that can be found on skin. Therefore all the equipment used in this part of the experiment was RNase free, and great care was taken to ensure no contamination occurred during the experiment. In addition the cells were keep on ice throughout the experimental protocol unless specifically stated.

A cell suspension of $3x10^6$ mononuclear cells per ml of culture medium was obtained from peripheral blood as previously described. Solutions of KLH at a concentration of 10μg/ml, conA at a concentration of 6.6μg/ml and PPD at a concentration of 1000 international units/ml were prepared in culture medium. 1.5ml of cell suspension and 1.5ml of each antigen solution were cultured on separate wells of a 6 well flat bottomed plate for 20 hours at 37^0C in 5% CO_2 and 95% O_2.

The cells were removed from the well by a Pasteur pipette and placed in a sterile universal. The wells were rinsed with RPMI-1640 medium and a cell scraper was used to remove any adherent cells from the well which were added to the sterile universal. After a further wash, the cells were recovered by centrifugation at 500g for 8 minutes at 4^0C and resuspended in 0.5ml of RMPI 1640. 0.25ml of the cell suspension was placed in each of 2 large Eppendorf tubes and 0.75 ml of TRIzol® (GibcoBRL) was added to each tube. TRIzol® is a ready to use reagent, containing phenol and guanidine isothiocyanate, that disrupts cells membranes, dissolves cell components and maintains the integrity of RNA. The cells were lysed by repetitive pipetting.

0.2ml of chloroform was added to each tube, which were mixed vigorously to ensure a good emulsion and left to stand at room temperature for 2-3 minutes. The tubes were centrifuged at 12,000g for 15 minutes at 4^0C. The mixture separated into a lower phenol-chloroform

phase and an upper aqueous phase, in which the RNA was dissolved. The aqueous phase was removed and placed in a fresh Eppendorf.

RNA was precipitated from the aqueous phase by adding 0.5ml of isopropanolol. The tubes were centrifuged at 12,000g for 10 minutes at room temperature. The supernatant was discarded and the pellet, containing the RNA, was resuspended in 0.75 ml of 75% ethanol, prepared with RNase free water. The sample was mixed by vortexing and the RNA was re-precipitated by centrifugation at 7,500g for 5 minutes at 4^0C. Ethanol dissolved any residual proteins, which were discarded by removing the supernatant, and thus this step cleaned the RNA. The tubes were vacuum dried for 4-5 minutes to remove any residual ethanol.

Diethylpyrocarbonate (DEPC) treated water was prepared in advance by adding 0.01% vol/vol DEPC to water in an RNase free glass bottle, allowing the mixture to stand overnight and autoclaving. 5µl of DEPC treated water was added to each Eppendorf, and the mRNA was dissolved by heating the tubes to 55-60^0C in a water bath for 10 minutes.

To calculate the quantity of RNA obtained, 1µl of RNA solution was added to 499µl of DEPC treated water. The OD of the solution was determined at 260nm, and the quantity of RNA in µg/ml or nanograms (ng)/µl was calculated using a standard correction factor of 40.

3.7.2.2 Formation of complimentary deoxyribonucleic acid (cDNA) by Reverse Transcription

3µg of RNA was transferred to a fresh small Eppendorf tube and the volume was made up to 11µl by adding the required amount of DEPC-treated water. 1µl of oligo-dT primer (GibcoBRL) was added, and the tube was heated to 70^0C for 10 minutes. All mRNA has a poly-A tail, to which the oligo-dT primer will bind at this temperature.

The tubes were cooled on ice for three minutes, and the solution was collected at the bottom of the Eppendorf by microfuging each tube for a few seconds. 4µl of strand buffer, containing 0.25M Tris HCl (pH 8.3), 0.375M KCl and 15mM $MgCl_2$, were added to each

tube, along with 1μl of nucleotide solution (dNTP) in which each of the nucleotides were present at a 10mmol concentration, 2μl of 0.1M dithiothreitol (DTT) and 1μl of Maloney Leukaemia Virus (MMLV) enzyme (all GibcoBRL). MMLV enzyme is a reverse transcriptase enzyme isolated from the Maloney Leukaemia Virus. The tubes were incubated at room temperature for 10 minutes and then at 37^0C for 1 hour. The experimental protocol was designed to allow the oligo-dT primer to bind to all RNA sites with a poly-A sequence, such as found at the end of all mRNA tails. The MMLV reverse transcriptase transcribes cDNA from the mRNA starting at sites where the oligo-dT primer has bound. Thus all mRNA should be converted to cDNA. At the end of the incubation the tubes were heated to 90^0C for 10 minutes, which denatures the MMLV enzyme.

The tubes were placed on ice for 10 minutes. 1μl of ribonuclease H (3.0 units/ml – GibcoBRL) was added and the tubes were incubated at 37^0C for 20 minutes. Ribonuclease H breaks down any mRNA in the sample, including any that is in double stranded form with its cDNA, thus leaving pure cDNA in the sample. The samples were frozen at -20^0C until use.

3.7.2.3 Polymerase Chain Reaction (PCR)

The technique of PCR is designed to amplify small quantities of specific DNA, identified by complimentary primers to that sequence, by using a DNA polymerase enzyme to copy the DNA many times. Each cycle of PCR doubles the quantity of specific DNA and thus the total quantity of DNA rises exponentially. Thus even minor contamination of the samples can lead to very misleading results. To prevent contamination a dedicated PCR room was used, separate flow cabinets were used for the steps before and after adding cDNA, and displacement pipettes were used to prevent aerosols formation.

PCR assays were developed to measure mRNA for actin, IL-2, IL-4, IL-10 and IFN-γ. The PCR conditions were based on, and primer sequences obtained from, the methods used by Jarvis *et al*[182]. The following solutions were prepared:

- doubly distilled water (DDW).
- standard buffer (x10 strength) which contained 200mM Tris-HCl (pH 8.4) and 500mM KCl.
- dNTP solution in DDW containing each nucleotide at a concentration of 5mM.
- 50mM magnesium (GibcoBRL).
- Primers specific for the cytokine cDNA (Oberon) were dissolved in DDW to give a 10mM solution. The primer sequences were those used by Jervis *et al*[182]. The primer sequences are shown in table 3.2.
- Taq DNA polymerase isolated from *Thermus aquaticus* YT1 (GibcoBRL) at a concentration of 5units/μl.
- cDNA prepared from the cell culture as described above.

The required volumes of standard buffer, dNTP, primers, magnesium solution and DDW were mixed. Different quantities of each of these solutions were required for each cytokine PCR assay, and the exact volumes of each constituent used in the different assays are shown in table 3.3. The tubes were moved to a separate flow cabinet and 1μl of cDNA was added. 50μl of paraffin oil was layered on top of the solution in each tube, which were then heated on a heater block to 94^{0}C for one minute, before being cooled to 80^{0}C. 1μl of Taq DNA polymerase was added to each tube. The contents were mixed in a shaker and the liquid was pooled at the bottom of the tube with a pulse of centrifugation. This "hot start" technique was designed to increase the sensitivity of the assay.

The heater block was programmed. Firstly, it was heated to 94^{0}C for 1 minute, which denatured (i.e. reduced double-stranded DNA to single stranded form) and uncoiled the DNA. Next it was cooled to 58-60^{0}C for 30 seconds, at which temperature the primers annealed to the complimentary DNA. Lastly it was heated to 72^{0}C for 45 seconds. At this temperature, Taq DNA polymerase will bind to any double stranded DNA, such as that marked by the primers, and replicate the downstream DNA strand. The program was

repeated from 33-35 cycles, depending on the assay. The PCR products were stored at 4^0C until use.

A sample containing no cDNA was run with each PCR assay performed. This acted as an important negative control to exclude contamination of the assay by exogenous DNA.

3.7.2.4 Analysis of PCR Products

3.7.2.4.1 Preparation of Acrylamide Gel

A 9% wt/vol acrylamide gel was prepared from the following reagents:

- 10xTBE containing 1M Tris, 0.9M boric acid and 0.01M EDTA (GibcoBRL). A 1/10 dilution of 10xTBE with distilled water gave a 1xTBE solution.
- 30% wt/vol acrylamide in distilled water (GibcoBRL).
- N,N,N',N'-tetramethylethylenediamine (TEMED) (GibcoBRL)
- 25% wt/vol of ammonium persulfate (APS) in DDW

6ml of DDW, 3ml of acrylamide and 1ml of 10xTBE were mixed in a plastic universal. 50µl of 25% wt/vol APS and 15µl of TEMED were added and mixed well. The APS and TEMED polymerise acrylamide and set the gel in 3-5 minutes. While it was liquid, the solution was injected between two glass plates held 1mm apart by dividers. A comb was inserted at the top of the gel to create lanes for the samples and the gel was allowed to set.

Table 3.2 – Primer sequences used in the PCR assays. The primer sequences used to detect cytokine specific cDNA are shown. The primer sequences are those used by Jarvis *et al.*

Product	Direction	Sequence	Size (base pairs)
Actin	**5' prime**	**ATC TGG CAC CAC ACC TTC TAC AAT GAC CTG CG**	**838**
	3' prime	**CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC**	
IL-2	**5' prime**	**CAT TGC ACT AAG TCT TGC ACT TGT CA**	**305**
	3' prime	**CGT TGA TAT TGC TGA TTA AGT CCC TG**	
IL-4	**5' prime**	**CGG CAA CTT TGA CCA GGA CAC GGA CAC AAG TGC GT**	**344**
	3' prime	**ACG TAC TCT GGT TGG CTT CCT TCA CAG GAC AG**	
IL-10	**5' prime**	**AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG**	**328**
	3' prime	**AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG**	
IFN-γ	**5' prime**	**GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC**	**427**
	3' prime	**CTC CTT TTT CGC TTC CCT CTT TTA GCT GCT GG**	

Table 3.3 – PCR assay conditions for amplifying cytokine cDNA. The PCR assay conditions used in each cytokine specific PCR assay are shown. The assay conditions are adapted from Jarvis *et al.*

	Actin	*IL-2*	*IL-4*	*IL-10*	*IFN-γ*
DDW (µl)	24	24	22	24	24
Buffer (µl)	5	5	5	5	5
dNTP (µl)	8	8	8	8	8
Primer 5' (µl)	2.5	2.5	2.5	2.5	2.5
Primer 3' (µl)	2.5	2.5	2.5	2.5	2.5
Mg (µl)	2	2	4	2	2
cDNA (µl)	1	1	1	1	1
Taq polymerase (µl)	5	5	5	5	5
Annealing temp (^{0}C)	60	60	59	60	60
No. of cycles	33	33	35	33	33

3.7.2.4.2 Separation of PCR Products on Acrylamide Gel

10μl of each cDNA sample was mixed with 5μl of a loading stain of xylene cyanol and bromophenol blue dissolved in a glycerol loading buffer. Each sample was added to a lane of the acrylamide gel. A DNA ladder (GibcoBRL) made of fragments of DNA of known size was run on each gel.

A 10 amp electric current was passed through the gel. cDNA, being negatively charged, was attracted to the positive electrode and the speed at which it passed through the gel corresponds to the size of the cDNA fragment. Xylene cyanol also ran through the gel at the speed equivalent to a strand of DNA of 75 base pairs. When this stain reached the bottom of the gel, which took 90-110 minutes, the current was switched off.

3.7.2.4.3 Quantification of PCR Products

The gel was placed in a bath of 200ml of 1xTBE and 20ml of ethidium bromide on a rocker at room temperature. Ethidium bromide will bind to DNA. After 4-5 minutes, the TBE and ethidium bromide was drained off and the gels were washed on a rocker with 200ml of 1xTBE for a further 2-3 minutes to remove any excess ethidium bromide.

The gel was placed under an ultraviolet light source attached to a computer running the molecular analyst program. Ethidium bromide fluoresces under ultraviolet light and so reveals the cDNA bands. The size of each band could be deduced from its position compared to the DNA ladder, and could be compared to the expected size of DNA fragment (see table 3.2). The molecular analyst program will calculate the degree of fluorescence from each band on the gel which should correlate to the amount of cDNA within each band.

3.8 Flow Cytometry Analysis of Cell Surface Markings

3.8.1 An Introduction to the Principles of Flow Cytometry

Flow cytometry is a method of analysing physical characteristics of cells in suspension by focusing laser light onto a single cell at a time. Sensors measure the scatter of the laser light by the cell. The forward scatter measures cell size whereas the side scatter gives a measure of cell granularity. Therefore the physical characteristics of individual cells can be measured and recorded. Furthermore, if the cells are appropriately stained, the cells will fluoresce under laser light. Using fluorescent labelled monoclonal antibodies that recognised specific cell surface markers, one can also obtain data on the expression of these call markers on that cell. The data for all these variables can be stored and processed by computer, allowing one, for example, to assess the physical characteristics of cells expressing specific cell surface markers.

3.8.2 Monoclonal Antibodies Used in Flow Cytometry Experiments

Mouse monoclonal antibodies against desired human molecules were purchased from Serotec Ltd. Details on the composition and prepatation of each antibody are shown in table 3.4. All antibodies contained 0.1% sodium azide and 1% bovine serum albumin to act as preservatives and stabilisers. All reconstituted antibodies were stored at $4^{O}C$ until use.

Table 3.4 – Details of the conjugated monoclonal antibodies used in flow cytometry experiments. PE=phycoerythrin, FITC= fluorescein isothiocyanate

Antibody	Isotype	Clone Number	Preparation	Conjugate
Anti CD4	Mouse IgG1	RPA-T4	Dissolve in 1ml distilled water	PE
Anti CD8	Mouse IgG1	LT8	Dissolve in 1ml distilled water	PE
Anti CD25	Mouse IgG1	M-A251	Comes as liquid	FITC
Anti CD45RO	Mouse IgG2a	UCHL1	Comes as liquid	FITC
Anti CD38	Mouse IgG1	AT13/5	Comes as liquid	FITC
Anti HLA-DR	Mouse IgG1	B-F1	Comes as liquid	FITC

3.8.3 Cell Preparation, Staining and Use in Flow Cytometer

Mononuclear cells were prepared from peripheral blood, as described previously, and diluted to $3x10^6$ cells/ml of culture medium. KLH was prepared in a concentration of 10μg/ml of culture medium. 1.5ml of cell suspension and 1.5ml of KLH solution were incubated together in a 6 well flat-bottomed plate for 92 hours at $37^{0}C$ in 95% O_2 and 5% CO_2.

250μl aliquots of cell suspension were placed into individual tubes. The cells were pelleted by centrifugation at 400g for 8 minutes and resuspended in 1ml of PBS. 10μl of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody and 10μl of phycoerythrin (PE)-conjugated monoclonal antibody were added to each tube in the combinations shown in table 3.4 and incubated for 30 minutes at $4^{0}C$. The cells were washed twice with PBS to remove

any excess unbound antibodies, recovered by centrifugation at 400g for 8 minutes and resuspended in 0.5 ml of PBS containing 1% vol/vol BSA and 0.1% wt/vol sodium azide.

The flow cytometer was set to analyse the 2-colour fluorescence of FITC and PE and record forward and side scatter of light. 10,000 cells from each sample were run through the machine and the data stored on disc and analysed using the CellQuest analysis program.

Table 3.5 – Combination of cell surface markers used in flow cytometry experiments. The cell cultures were incubated with the above combination of monoclonal antibodies prior to being analysed by flow cytometry. The negative controls consisted of a mouse monoclonal antibody conjugated to the appropriate fluorescein which does not bind to human cells will therefore give information on the levels of non-specific binding of these monoclonal antibodies to human cells.

	Fluorescent Stain	
	PE	FITC
Target of Monoclonal Antibody	Negative Control	Negative Control
	CD4	CD25
	CD4	CD38
	CD4	CD45RO
	CD4	HLA DR
	CD8	CD25
	CD8	CD38
	CD8	CD45RO
	CD8	HLA DR

3.8.4 Analysis of FACS Scanning Data

Analysis of the data was performed using the CellQuest computer program. The program was instructed to identify lymphocytes on the basis of their size (forward scatter) and granularity (side scatter). Subsequent analysis was performed on these cells. The degree of staining from the FITC labelled antibody bound to each activation marker and PE labelled antibody bound to CD4 or CD8 was recorded. The amount of stain correlates to the amount of cell surface marker present. The degree of staining of activation marker was plotted against the degree of staining of CD4 or CD8. This dot-plot was divided into quadrants to indicate positive or negative staining. The level of staining required to denote a positive result was calculated from the amount of non-specific staining on cells incubated with

control antibody. The number of cells in each quadrant was calculated. Statistical analysis was performed on the sample from the volunteer prior to encounter with KLH compared to the sample after eating or after immunisation.

An alternative method of analysis used was to plot the frequency of cells that stained at different levels for the activation marker and compare the frequency distribution of cells in the samples taken before and after encounter with KLH. This method may demonstrate more subtle changes in the level of activation markers than that seen on the dot-plot analysis.

3.9 Statistical Analysis

Unless specifically stated in the text, staistical analysis was performed as follows. Mann-U Whitney testing was used to assess differences between continuous variables and Chi-squared testing was used to compare two qualatitive variables between groups.

4 DEVELOPMENT OF THE PROTOCOL AND ASSAYS

4.1 Introduction

To demonstrate oral tolerance, a safe immunisation schedule that provoked a systemic immune response had to be devised, and assays to measure this immune response had to be developed. This chapter will discuss the work I performed to develop and validate these assays.

4.2 Development of the Injection Schedule

4.2.1 Work to Establish the Safety of KLH and OVA for Immunisation

KLH has a long history of being used as a systemic immunogen in humans[183], and has also been fed to humans[9]. The World Health Organisation (WHO) has recommended the use of KLH as an antigen for the investigation of immunodeficiency states[184]. There have been no reported side effects of KLH in humans. Thus KLH appears to be safe for use in humans. OVA is a common dietary antigen, to which most people have had prolonged exposure. It has been used as an immunogen in many animal experiments, and appears to be safe.

KLH and OVA were manufactured by Sigma-Aldrich Co. to American Food and Drug administration standards, which ensures that no contamination occurred during the manufacturing process. To ensure no contamination occurred, the preparation of the immunisations was undertaken by the Western General Hospital aseptic pharmacy department. Once dissolved, the solutions were passed through a 0.45μm filter (Millipore) to remove any large insoluble particles, including most bacteria, diluted and stored at 200μg/ml at 4^0C.

Before the start of the project, two test samples were cultured by the bacteriology department. Both samples demonstrated no growth. Samples were also tested for endotoxin,

using an established ELISA-based method. Three endotoxin units per ml for KLH solution and 10 endotoxin units per ml for the OVA solution were detected. These are well within the safety limits for human injection recommended by the American Association of Pharmacists[185]. These data suggested that the preparations of KLH and OVA were safe to give systemically to humans.

4.2.2 Development of Immunisation Schedule

To demonstrate the induction of tolerance, it was necessary to induce a positive immune response in the control group. KLH was given at different doses and at different times in order to find the optimal immunisation schedule. It was found that two injections of 200μg of KLH given 10 days apart induced a positive DTH response three weeks after the initial immunisation, and this immunisation schedule was used in all experiments using KLH.

4.3 Development of the *In Vitro* Lymphocyte Proliferation Assay

4.3.1 Isolation of Cells

The technique used for isolating mononuclear cells worked reliably. 1-3 x 10^6 mononuclear cells were isolated per ml of blood venesected. Several samples were tested for contamination with non-mononuclear cells on light microscopy. The samples tested contained 0-5% polymorphonuclear granulocytes and 0-1% red cells.

4.3.2 Lymphocyte Proliferation Assay

A series of experiments were performed to determine the optimal conditions of the lymphocyte proliferation assay. The assay conditions were altered to determine those that gave the lowest result for the control wells and the highest results for those stimulated with antigen. The assay conditions that were tested included the concentration of KLH used (test

range of 1-100μg/ml), the type of FCS used, the length of time in culture (from 48 to 96 hours), and the length of time that ^{3}H-thyimidine should be added before the end of culture (6-18 hours). The conditions that gave the optimal results are those described in the materials and methods section. Using these methods, the mean result for the negative controls (i.e. cells cultured with culture medium alone) was 998.8 counts per minute with a standard deviation of 812.8.

4.4 Development of KLH ELISA

4.4.1 Introduction - Problems and Standards

I developed the KLH ELISA *de novo*. A significant problem was that I had no known positive control with which to establish the ELISA. To overcome this problem I used sera taken from volunteers who had had trial immunisation with KLH as detailed above. In addition, one volunteer, who had had only one injection with KLH initially, received a second injection seven weeks later. His serum had a moderately high level of anti-KLH antibodies and was used to optimise this ELISA in the experiments detailed below.

4.4.2 Experiments to Establish ELISA Technique

Experiments were conducted to test all aspects of the assay, and the conditions chosen were those that gave the highest values for the post-immunisation samples and the lowest values for the control and the pre-immunisation samples. Variables that were tested included the coating concentration of KLH (ranging from 100μg/ml to 0.1μg/ml), the assay diluent (bicarbonate buffered saline against PBS), the blocking solution (BSA, adult bovine serum and FCS were all tested at concentration of 1% vol/vol or 5% vol/vol) and the type of plate used (immulon 1® or immulon 2®). The optimal time for the coating, blocking and conjugation stages of the assay were also assessed. The optimal concentration of serum (test

dilutions ranged from 1:50 to 1:3200) and of conjugated antibody (from 1:500 to 1:8000) were tested.

The optimal results were obtained using the methods described in the materials and methods section.

4.4.3 Validation of the anti-KLH IgG and IgA ELISA Assays

The within plate co-efficient of variance (C.V.) (=(standard deviation / mean)x100) was calculated for the anti-KLH IgA and IgG ELISAs. Two samples, one with high concentrations of both antibodies and one with low concentrations of both antibodies were each put in 40 wells of a plate, which was processed by the normal ELISA methods descried above. The within plate C.V. of the anti-KLH IgG ELISA was 9.4% at a low value of 160 units, and 16.1% at a high value of 550 units. The within plate C.V. of the anti-KLH IgA ELISA was 9.2% at a low value of 360 units and 3.5% at a high value of 3800 units.

The between plate variation of the ELISA was also calculated based from several samples run on two plates. The mean variation of these samples between plates was 27.5% for the anti-KLH IgG assay and 27.8% for the anti-KLH IgA assay. Although these results are satisfactory, to avoid the increased error of running samples on separate plates, all post-immunisation samples of both the fed and control groups were run on one plate.

The difference between each serum sample run on the same plate as duplicates were within 10% for the IgG assay, and for the IgA assay three pairs were over this range, but were all within 12.5%.

On each plate a standard curve was calculated using doubling dilutions of serum taken from a volunteer after receiving a third immunisation 7 weeks following the second immunisation. The starting dilution was 1:50 and ran to 1:3200. The standard curve was calculated using linear regression on a logarithmic/linear scale for IgG. The r-value for fit of the standard curve produced was between 0.978 and 0.984. For IgA, quadratic regression was used to obtain the standard curve, and the r-value exceeded 0.999. Examples of typical standard

curves obtained are shown in figures 4.1a and 4.1b. The positive control sample was arbitrarily ascribed a value of 1600 units of both anti-KLH IgG and IgA. Several test samples were above the upper limit of the reference range of the standard curve. These samples were rerun at a 1:800 dilution to allow the data to be entered into the appropriate equation.

The above data suggests that these ELISAs are reliable and reproducible.

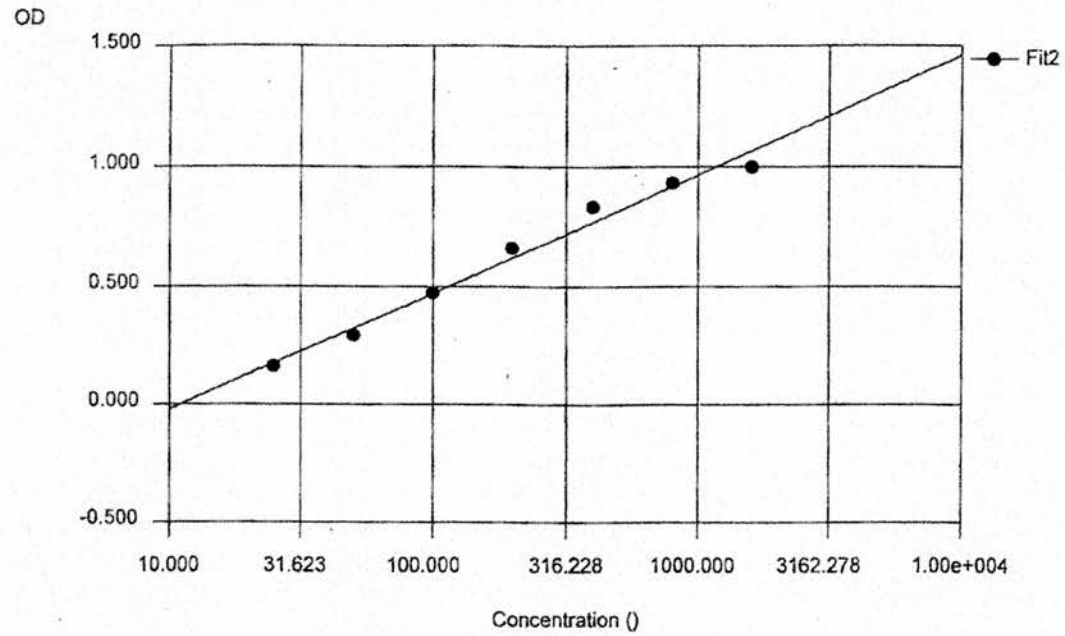
4.4.4 Validation of anti-KLH IgG1 and IgG2 ELISAs

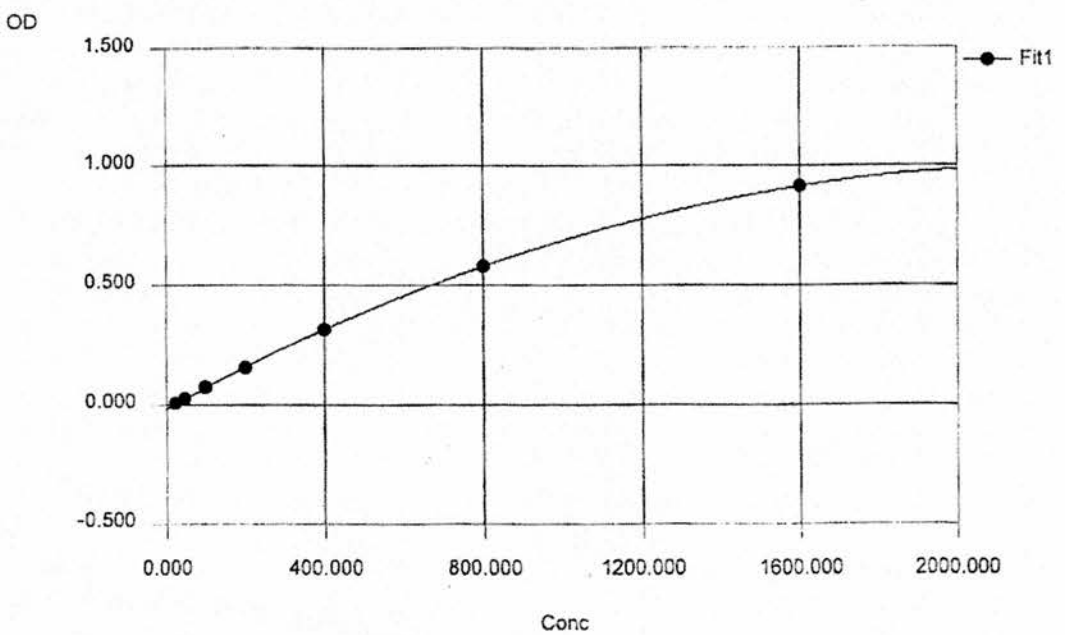
Mouse anti-human IgG1 and IgG2 antibodies were purchased and used to adapt the anti-KLH IgG ELISA to assay these two subtypes. The within plate C.V. was very good. For the IgG1 assay, the C.V. was 7.7% at a value of 550 and 5.6% at a value of 1950. For the IgG2 assay, the C.V. was 9.8% at a value of 540 and 9.8% at a value of 3580. The post-immunisation samples from all the groups were read on the same plate to remove inter-plate variability as a possible error.

4.4.5 Validation of the Anti-OVA IgG and IgA ELISA Assays

Previous work in the gastrointestinal laboratory has involved investigating humoral responses to OVA by quantitative isotype-specific ELISA methods, particularly in disease states. Therefore assay methods had been previous developed and validated, and these methods were used in my experiments. In my hands, the within plate coefficient of variation for the IgG ELISA was 10.6% for a reference specimen at a high value of 1020 units/ml and 13.8% for a specimen with a low value of 55 units/ml. For the IgA assay, the within plate C.V. was 12.0% at a value of 15 units/ml. All the samples that were compared were run on the same plate to remove the potential problems of inter-plate variation. In addition any samples that gave interesting or unusual results were repeated on a separate run to ensure that the results were reproducible.

Figure 4.1 – Typical standard curves obtained using the high standard as detailed above. OD is plotted on the Y-axis and anti-KLH antibody is plotted on the X-axis. Figure 4.1a shows a typical standard curve for IgG and figure 4.1b shows a typical standard curve for IgA. The mean OD for each test sample is read off the Y-axis and the value in standard units is derived from the corresponding value on the X-axis.





5 RESULTS OF EXPERIMENTS USING KLH TO INDUCE ORAL TOLERANCE

5.1 Introduction

In this section, I report the results of the experiments used to demonstrate oral tolerance to a neoantigen in humans. The protocol I used was based on that of Husby and colleagues and has been described in the Materials and Methods section. In summary, a control group was immunised with subcutaneous KLH. The immune responses to KLH were measured by DTH responses to intra-dermal KLH, *in vitro* lymphocyte proliferation to KLH and KLH specific IgA and IgG antibody responses.

KLH was fed to test groups starting 21 days before they received the same immunisation schedule that was given to the control group. Three different feeding regimes were used; namely 10mg of oral KLH given daily for 10 consecutive days (KLH-fed group 1), 50mg of KLH given daily for 10 consecutive days (KLH-fed group 2) and 50mg of KLH given for 15 days in a divided course (KLH-fed group 3). Any differences between the immune responses in the fed groups and the control group were attributed to the effect of oral tolerance.

The exact timing of the feeding regime, immunisation schedule and immunological testing are shown in table 2.1 in the Materials and Methods section.

5.2 Results

5.2.1 Delayed Type Hypersensitivity Responses

Intradermal administration of antigen led to a DTH response characterised by erythema, swelling and induration at the injection site and is an *in vivo* measure of cell-mediated immunity. Two methods of measuring the DTH response were employed. Table 5.1 shows the results of DTH response as assessed by the presence or absence of induration in the four groups and table 5.2 gives the results for the mean diameter of induration of the DTH response. Photographs 5.1 and 5.2 show typical positive and negative DTH responses at 24 hours.

Table 5.1 – results for presence of DTH responses in pre-fed and control groups after completion of the immunisation schedule. A positive DTH response was taken as the presence of any detectable induration. Statistical significance was determined by Fisher's exact test. There was a significant reduction in positive DTH responses in the two groups pre-fed the higher doses of KLH than in the control group.

Group	No. of positive DTH responses / No. in group		Statistical difference from control group	
	24 hours	48 hours	24 hours	48 hours
Control group (n=13)	11 / 13	10 / 13	Not applicable	
KLH-fed group 1 (n=4)	4 / 4	2 / 4	N.S.	N.S.
KLH-fed group 2 (n=7)	2 / 7	0 / 7	$P<0.05$	$P<0.005$
KLH-fed group 3 (n=6)	2 / 6	0 / 6	$P<0.05$	$P<0.005$

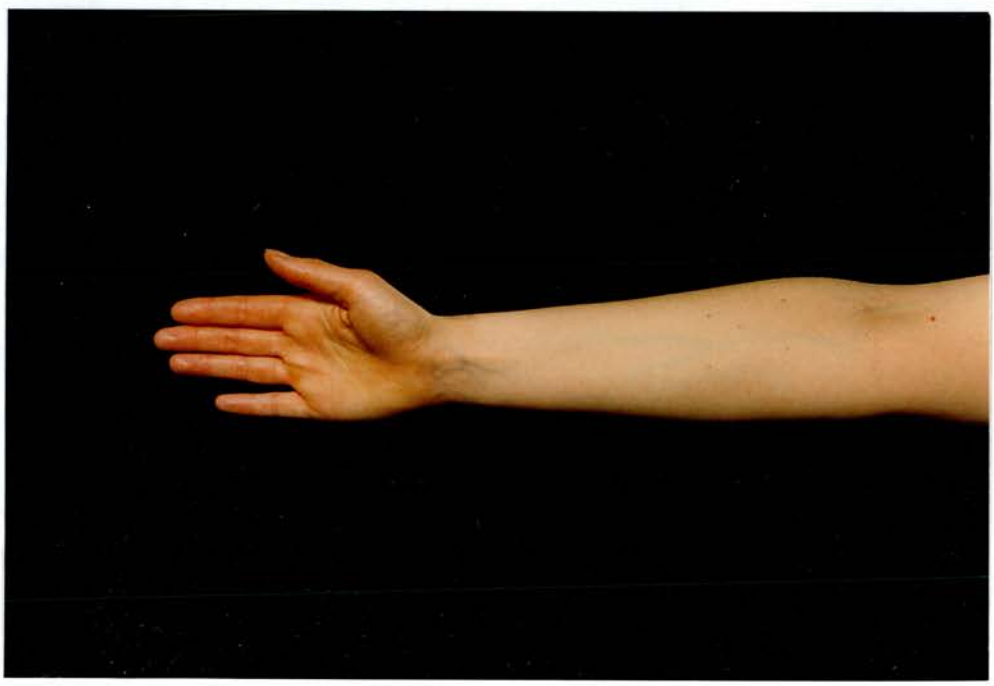
Table 5.2 – results for DTH responses as measured by the diameter of erythema following intra-dermal injection of KLH in pre-fed and control groups after completion of the immunisation schedule. There was a significant reduction in the diameter of erythema in the two groups fed the higher doses of KLH compared to the control group at 48 hours.

Group	Median diameter of erythema (mm) (Range/1st quartile/3rd quartile)		Statistical difference from control group	
	24 hours	48 hours	24 hours	48 hours
Control group (n=13)	22 (0-38/14.25/28.25)	15.5 (0-35/8.5/24.25)	Not applicable	
KLH-fed group 1 (n=4)	20.5 (18-32.5/18/32.37)	4.2 (0-52/0/41.1)	N.S.	N.S.
KLH-fed group 2 (n=7)	10 (0-32.5/0/19)	0 (0-29/0/0)	N.S. (p=0.1)	P<0.05
KLH-fed group 3 (n=6)	0 (0-24/0/21.5)	0 (0-21.5/0/0)	N.S. (p=0.07)	P<0.05

The results of the DTH responses show that the KLH immunisation schedule used caused a positive DTH response in the majority of volunteers. There was a significant reduction in DTH responses after immunisation in the two groups that were pre-fed the higher doses of KLH prior to immunisation. The group that was fed the lowest dose of KLH showed no significant reduction in DTH responses compared to the control group, although the numbers in this group were small.

Photo 5.1 – A typical positive DTH response is shown. This photo was taken 24 hours after the intra dermal injection was given. Marked erythema and induration can be seen.

Photo 5.2 – A typical negative DTH response is shown 24 hours after a prefed volunteer (in this case from KLH-fed group 3) was given the intra-dermal KLH injection. The remains of the white bleb from the injection can be seen.



5.2.2 *In vitro* Lymphocyte Proliferation Responses

In vitro lymphocyte proliferation was the second measure of CMI used in this study. Figure 5.1 shows the results of the lymphocyte proliferation responses in the control and pre-fed groups as expressed by the lymphocyte proliferation index before and after immunisation, and (where appropriate) before feeding.

The results showed that there was no antigen specific lymphocyte proliferation before KLH exposure in any group. The immunisation schedule caused a significant increase of lymphocyte proliferation in the control group ($p=0.0001$ compared to pre-immunisation values). Pre-feeding 10mg of KLH for 10 days (KLH-fed group 1) caused no significant lymphocyte proliferation but subsequent immunisation again resulted in a positive lymphocyte proliferation response in the one subject in which the assay did not fail for technical reasons. Feeding either of the higher doses of KLH (KLH-fed groups 2 and 3) caused a significant rise in lymphocyte proliferation compared to baseline ($p<0.01$ for both groups). After immunisation there was a further significant rise in lymphocyte proliferation in KLH-fed group 2 ($p<0.005$). In KLH-fed group 3, a significant level of lymphocyte proliferation remained after immunisation ($p<0.005$ compared to baseline) but the level of lymphocyte proliferation was similar to the post feeding values (p=N.S.).

Figure 5.2 shows the same data expressed by time of testing rather than by group and without the data from KLH-fed group 1. It illustrates the following additional points. Firstly, the group fed most intensively had a significantly greater lymphocyte proliferation response before immunisation ($p<0.05$) but a significantly reduced degree of lymphocyte proliferation after immunisation compared to KLH-fed group 2 ($p=0.05$). The lymphocyte proliferation index of the control group after immunisation was less than group 2 and greater than group 3 but not statistically different to either.

Lastly figure 5.3 illustrates the relationship between the *in vitro* lymphocyte proliferation results and the DTH results at 24 hours (as calculated by the presence or absence of

Figure 5.1 – Lymphocyte proliferation responses for the control group and the KLH fed groups shown by group. The solid bars indicate the baseline values prior to KLH exposure, the striped bars show the results after completion of the course of KLH feeding and the checked bars demonstrate the post immunisation values. The standard error of the mean is shown by the error bars. There was no detectable lymphocyte proliferation prior to KLH exposure in any group. Immunisation with KLH in the control group caused a significant increased in lymphocyte proliferation compared to baseline ($p=0.0001$). Feeding alone caused a significant increase in the lymphocyte proliferation response in KLH groups 2 and 3 ($p<0.01$) but not KLH group 1. After immunisation, there was a further significant increase in lymphocyte proliferation in KLH group 2 ($p<0.005$), but not in KLH group 3. In KLH group 1, only 1 post immunisation sample gave a valid result (marked *).

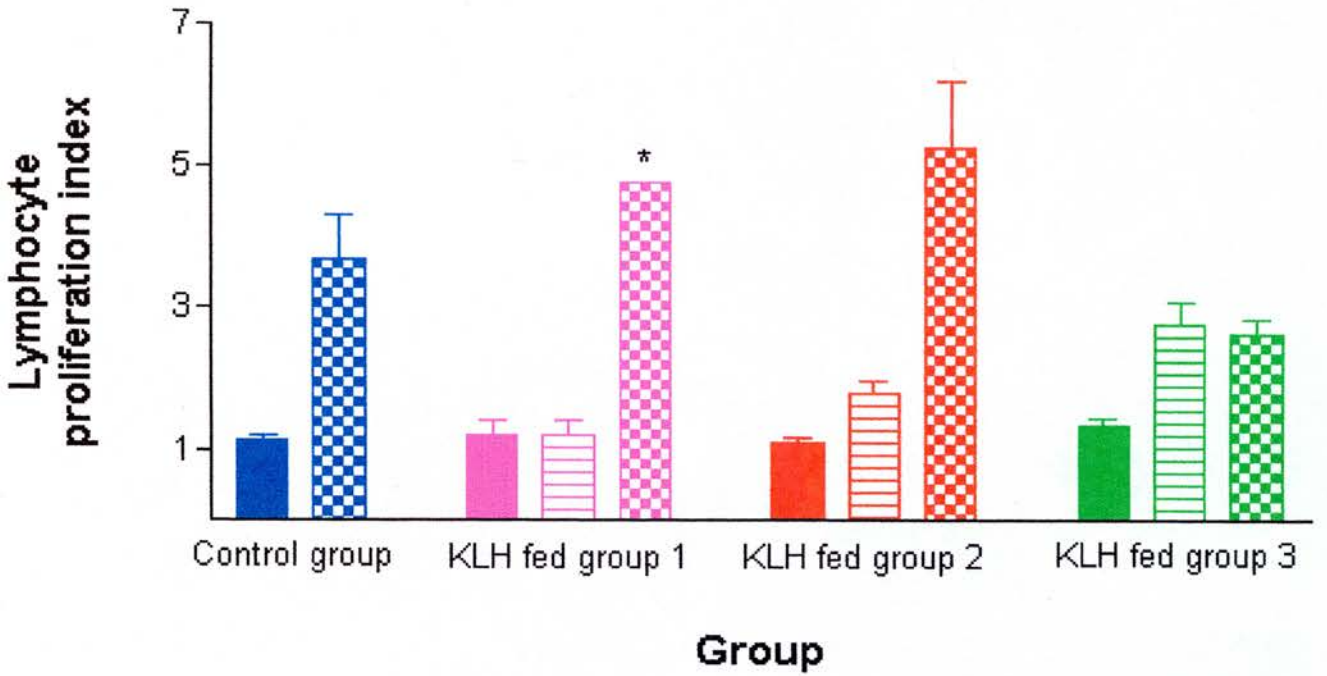


Figure 5.2 – Lymphocyte proliferation responses for the control group and KLH-fed groups 2 and 3 shown by time of sampling. The error bars represent the standard error of the mean. The results expressed in this way illustrate two additional points. Firstly, there is a significantly greater increase in lymphocyte proliferation after feeding in KLH-fed group 3 compared to KLH-fed group 2 ($p<0.05$). Secondly there is a significant reduction in lymphocyte proliferation after immunisation in KLH-fed group 3 compared to KLH-fed group 2 ($p=0.05$).

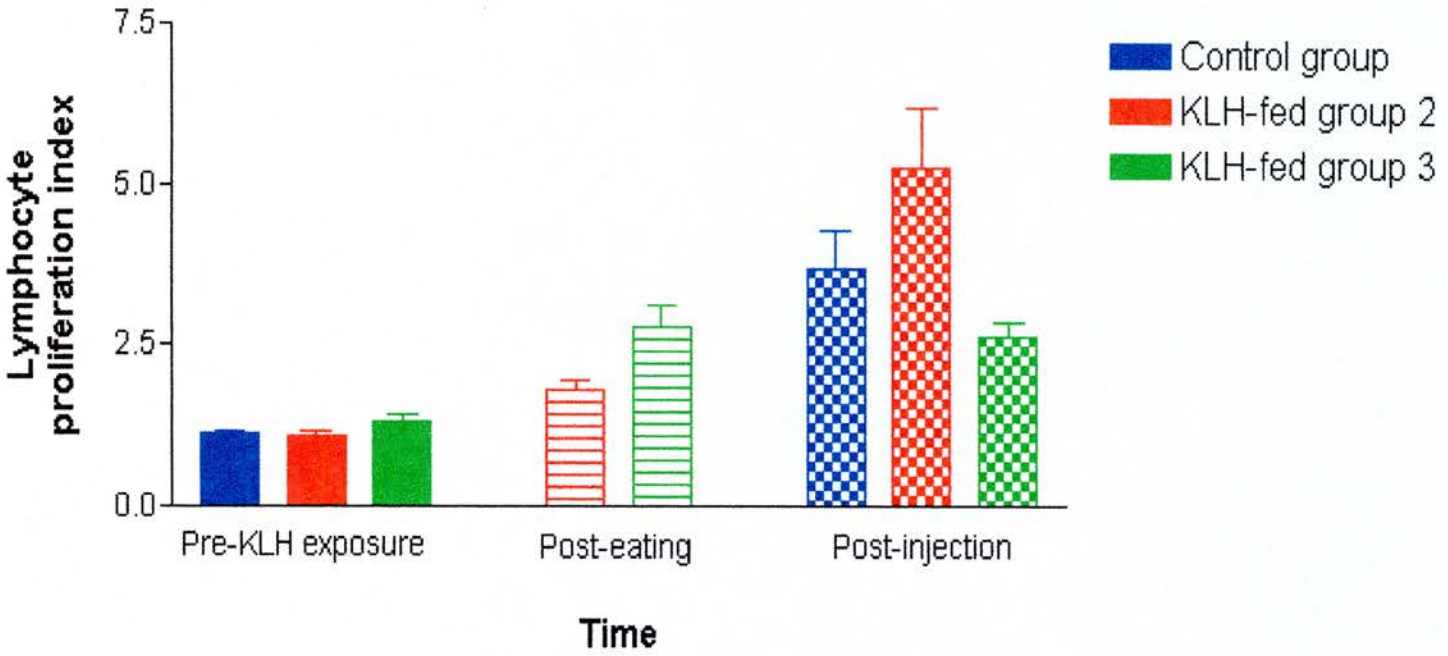
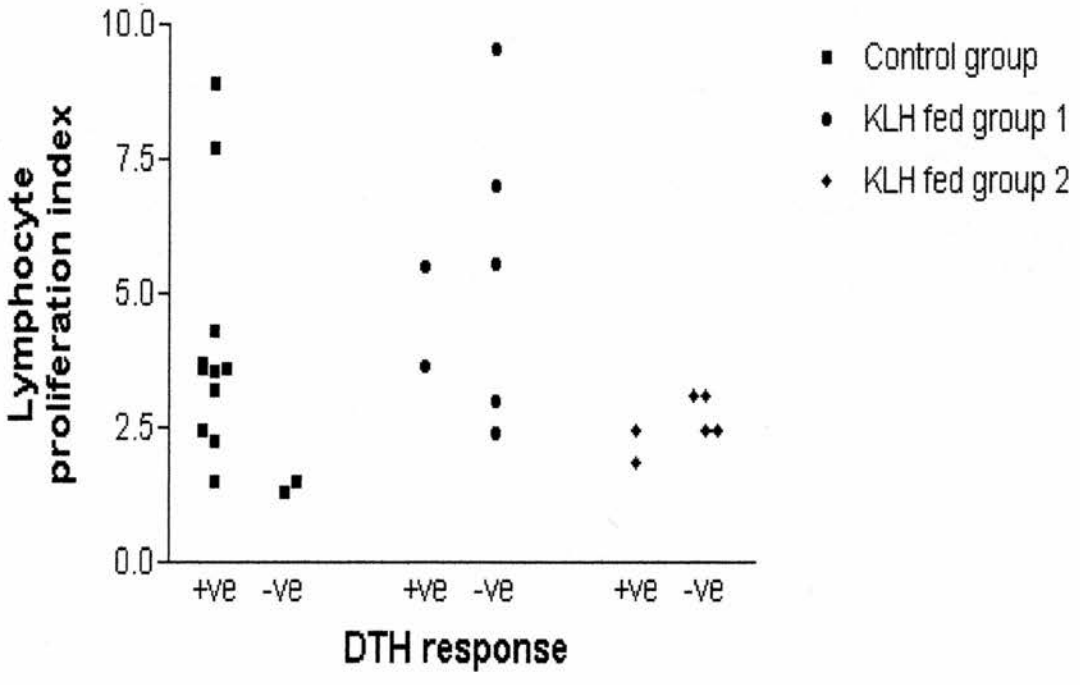


Figure 5.3 – Relationship between *in vitro* lymphocyte proliferation index results and *in vivo* DTH testing in the control group and KLH-fed groups 2 and 3. In the control group, there is a relationship between a negative DTH response, as defined by the presence of any detectable induration at 24 hours, and a low lymphocyte proliferation index. This relationship is lost in both KLH-fed groups 2 and 3.



induration) for the control group and KLH-fed groups 2 and 3. In the control group, the two volunteers without a positive DTH response also had the lowest levels of *in vitro* lymphocyte proliferation. There was no relationship between the DTH results and *in vitro* lymphocyte proliferation in either of the KLH-fed groups.

5.2.3 Antibody Response

The anti-KLH IgG and IgA antibody responses are shown in graphs 5.4a and 5.4b respectively. There was no detectable antibody response in any group before KLH exposure, as would be expected since KLH is a neoantigen. The control group showed a small, but significant ($p<0.001$) IgA response at 10 days (i.e. after the first immunisation with KLH), but no IgG response at that time. After completion of the immunisation schedule, at 21 days, there was a highly significant rise in both anti-KLH IgG ($p=0.0001$) and IgA ($p<0.0001$).

No anti-KLH IgG or IgA was detected after any of the KLH feeding regimes. The antibody responses following immunisation in KLH-fed group 1 were similar to the control group. In KLH-fed group 2 there was a statistically significant rise in anti-KLH IgG after immunisation ($p=0.02$), and a trend towards an increased response at 10 days ($p=0.07$) compared to the control group, suggesting that this course of feeding primed the IgG responses. There were no statistical differences in anti-KLH IgA levels compared to the control group at either time-point. The results for KLH-fed group 3 showed no significant rise in anti-KLH IgG compared to the control group. The anti-KLH IgA levels were also unchanged from the control group, although there may have been a trend towards reduced levels of serum anti-KLH IgA with increasing doses of feeding.

Figure 5.4a – Anti-KLH IgG responses shown by group.

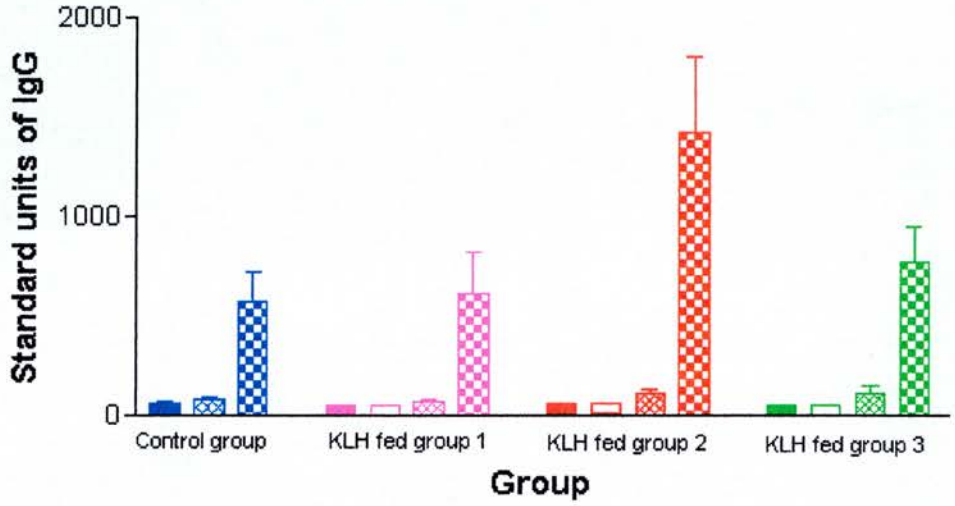
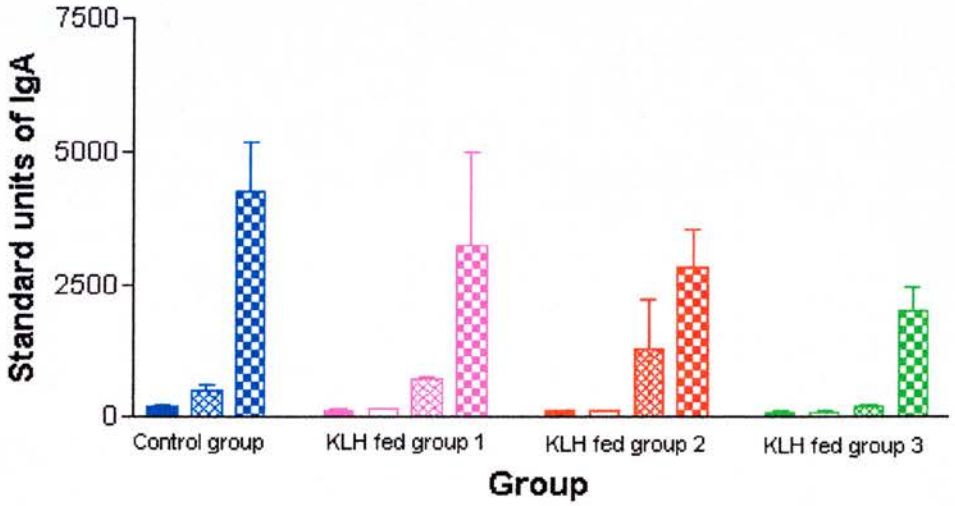


Figure 5.4b – Anti-KLH IgA responses shown by group.



The graphs illustrate the anti-KLH IgG and IgA responses by group at each time point tested. The solid bars show the results prior at baseline, the empty bars are after feeding KLH, the crossed bars are prior to the second KLH immunisation and the speckled bars are at the end of the protocol. The error bars represent the standard error of the mean. The results illustrate that immunisation with KLH causes both an IgG and IgA response. In KLH-fed group 2 there is a significant augmentation of the IgG ($p=0.02$), but not IgA, response after immunisation. This is not observed in KLH-fed group 3.

5.3 Discussion of Results

5.3.1 Rationale for Use of Keyhole Limpet Haemocyanin

KLH is obtained from the deep-sea crab Megalla crusatacean. This crab is not a normal constituent of the diet, and therefore KLH is a neoantigen i.e. most people have never had exposure to it. KLH is a large molecule weighing 50,800 daltons. Furthermore it can polymerise, increasing its molecular weight up to 27,000,000 daltons[186]. KLH has long been used as a systemic immunogen in humans[183], and has also been fed to humans[9]. There have been no reported side effects to KLH, and indeed the WHO recommends its use in the investigation of immunodeficiency states[184]. Thus KLH is a neoantigen, it has a record of safety in humans use, and a proven ability to provoke a good immune response in humans. For these reasons, it is an ideal antigen with which to investigate the immune responses to a new, orally encountered antigen in humans.

5.3.2 Assays Used to Measure the Immune Response

5.3.2.1 Delayed-Type Hypersensitivity Reactions

A DTH reaction is a complex immune response whereby antigen exposure results in an erythematous, inflammatory immune response caused by the infiltration of lymphocytes, macrophages and basophils at the site of antigen exposure over 24-48 hours. The DTH response is induced by Langerhan's cells which sample antigen in the epidermis and migrate to the dermis. At this site the antigen is presented to primed, antigen specific CD4+ cells which are activated and produce pro-inflammatory cytokines. Among these is IFN-γ, which activates keratinocytes to express intercellular adhesion molecule-1 and major histocompatibility class II and secrete IL-6 and GM-CSF. These factors recruit antigen non-specific CD4+ T cells and activated macrophages to the site of inflammation. The production

of a DTH response is a complex process, but does require the presence of antigen specific, IFN-γ producing CD4+ T cells. These cells used to be termed T_{DTH} cells, but are in fact morphological indistinguishable from other T_H cells and have been termed T_H1 cells in mice. Due to this complex pathway, DTH responses take 12 hours to become detectable, reach their peak at 24-36 hours and subside from 48 hours onwards. I chose to measure DTH responses at their peak (24 hours) and as they were starting to subside (48 hours). I made all the measurements of DTH responses to avoid inter-observer variation. Two separate measures of DTH response were made, namely induration and erythema. The method of using the presence or absence of detectable induration to measure DTH was that used by Husby and colleagues in their experiments[9]. The observations could be subject to observer bias and so the second method was employed to attempt to reduce this source of bias.

5.3.2.2 *In Vitro* Lymphocyte Proliferation Assay

The lymphocyte proliferation assay is a well-established *in vitro* technique of investigating CMI. The assay relies on the incorporation of tritiated thymidine into dividing cells that have been stimulated by antigen. The assay is said to rely on the presence of antigen specific T cells in the test sample. The results quoted in this thesis are the lymphocyte proliferation index, which is calculated by dividing the uptake of thymidine by the antigen-stimulated cells by the uptake in unstimulated cells. This is the most common way of measuring lymphocyte proliferation.

5.3.2.3 Humoral Responses

The humoral arm of the immune response produces antibody which can opsonise and neutralise foreign antigen. This limb of the immune response was assessed by measuring KLH specific IgG and IgA antibody production by ELISA, which is a well-established technique to measure antibody quantitatively.

It should be noted that the values of IgA and IgG are not directly comparable since the values of antibody given are not absolute values but are calculated by comparing the test sample with a standard sample which is ascribed an arbitrary concentration. Therefore, although the numbers (units) quoted for IgA are higher than those for IgG units, no conclusions can be drawn about the relative concentration of each Ig in the systemic circulation.

5.3.3 Discussion of the Immune Responses in Each of the Test Groups

5.3.3.1 Control Group

There is an active immune response to immunisation with KLH comprising both positive cell-mediated and humoral immune responses in the control group. The positive DTH response suggests that the immune response in the control group is based on a T_H1 type response.

CMI was assessed by *in vivo* skin testing and *in vitro* lymphocyte proliferation assay. In the control group the two assays correlated in that those with a positive skin test had a higher lymphocyte proliferation index. Conversely, the two volunteers who had a negative skin test also had the two lowest values for the lymphocyte proliferation assay. Thus, the *in vitro* lymphocyte proliferation assay is indeed a marker of CMI in the actively immunised control group.

Antibody response to KLH was induced beginning at 10 days with a significant rise in IgA levels compared to baseline. IgG levels were not significant elevated compared to baseline levels at 10 days. After 21 days (and a second injection), both anti-KLH IgG and IgA levels were greatly raised. The time scale of these responses was typical of a primary immune response.

In summary the KLH immunisation schedule evokes both cell-mediated and humoral immune responses.

5.3.3.2 KLH-Fed Group 1

There was no statistical difference in any measure of the immune response tested between the control group and KLH-fed group 1. Furthermore, there was no detectable immune response after feeding alone. Importantly, there were no side effects to either the KLH feeding or KLH immunisation. These results suggested that feeding this dose of antigen has no effect on the systemic immune system. Since oral tolerance can occur in humans[9], these results imply that a threshold of fed antigen may be required to induce this effect.

The dose of KLH used in the feeding schedule was lower than that used by Husby in the previously published trial, where 50mg feeds of KLH were given for 10 days[9]. Animal models suggest that the amount of antigen that needs to be ingested to induce tolerance varies depending on the type of antigen, but that in some models a lower dose of antigen per weight of animal than the dose of KLH used in my experiments on humans has induced tolerance[30]. This experiment was designed to investigate the effects of low dose feeding on oral tolerance induction in humans and was the principal reason for using such a low dose of feeding in these initial experiments. A secondary factor for using this low dose was the cost of KLH (£250 for 500mg).

In summary, 10mg of KLH fed for 10 days had no detectable effect on the subsequent systemic immune response to KLH immunisation and suggests that there is a minimal level of feeding that is required to induce oral tolerance in humans.

5.3.3.3 KLH-Fed Group 2

DTH responses to the immunisation schedule were significantly reduced by feeding 50mg of KLH for 10 days (see tables 5.1 and 5.2). The reduction appeared to be both in the absolute numbers that develop a response, as measured by induration at the site of injection, and the area of erythema. In addition there may have be an attenuation of DTH response since all the subjects in whom induration could be detected at 24 hours had a negative response at 48 hours (an observation that also applied to KLH-fed group 3), whereas only 1 patient in the

control group had a positive response that did not persist for 48 hours. These results suggest that oral tolerance for DTH had been induced in KLH-fed group 2.

The *in vitro* lymphocyte proliferation assay showed a positive response after feeding alone (or before immunisation), which suggests that antigen had been absorbed and recognised by the gastrointestinal immune system. After immunisation, there is a further significant increase of *in vitro* lymphocyte proliferation. The concordance between DTH responses and *in vitro* lymphocyte proliferation responses that was seen in the control group was lost (as was also the case in KLH-fed group 3). These results imply that pre-feeding antigen stimulates the production of a cell type that is activated in the lymphocyte proliferation assay, but does not initiate the DTH response.

The antibody results showed priming of IgG, but not IgA, responses by pre-feeding KLH. Thus, the cell that is activated by feeding appears to modify the systemic humoral immune response after immunisation.

This combination of results is consistent with the hypothesis that a regulatory cell was induced by feeding alone and could be detected in the lymphocyte proliferation assay. This cell modified the immune responses to the subsequent systemic exposure of antigen in such a way as to reduce *in vivo* DTH responses but to increase IgG humoral responses.

5.3.4 KLH-Fed Group 3

5.3.4.1 Introduction – Rationale for Feeding Schedule

The results that I obtained from the KLH-fed group 2 showed many similarities to the immune response observed by Husby[9]. In particular, DTH responses were reduced and IgG responses were primed. However, Husby observed that after immunisation, *in vitro* proliferation responses were significantly reduced, whereas in my experiments the (non-significant) trend was in the opposite direction.

One possible explanation for these differences is that the exact timings of feeding and immunisation were different and that although the regulatory cells induced by feeding was the same in both studies, Husby *et al* were unable to demonstrate lymphocyte proliferation because the antigen specific cell was not in the peripheral blood at the time of testing. The exact timings of the feeding schedule are shown in table 5.3. Alternatively, the differences may have been caused by an increased production of this regulatory cell as a result of the increase length of feeding used by Husby, which in turn further altered the balance of the immune responses away from an active responses, thus reducing lymphocyte proliferation. A third possible explanation is that the increased length of feeding caused the induction of additional, or more powerful, mechanisms of tolerance. Animal models have demonstrated multiple mechanisms of tolerance induction that can be induced by different doses of feeding[152]. Lastly, it is possible that the differences between these two results may be due to methodological differences or statistical chance, although this is less likely because in one case there is a significant reduction in lymphocyte proliferation, whereas in the other there is a trend towards increased proliferation.

To attempt to differentiate between these possibilities, I elected to feed a third group of volunteers an increased course of KLH comprising of 15 feeds of 50mg of KLH, given in two blocks over 3 weeks. If differences in the assay technique were responsible, the results should be similar to KLH-fed group 2, whereas, if additional, or more powerful, mechanisms of tolerance were induced by increasing the fed dose, one would expect the results in this group to mirror those of Husby more closely.

Table 5.4 – Comparison of the schedules used to demonstrate oral tolerance in humans in my experiments (top row) and Husby's experiments (bottom row).

Rbj	F	F	F	F	F	F	F	F	F	F					F*
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Hus	F	F	F	F	F										F

Rbj	F*	F*	F*	F*			I								
Day	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Hus	F	F	F	F							I				

Rbj		I											D
Day	31	32	33	34	35	36	37	38	39	40	41	42	43
Hus						I							D

Notes - 1) F = feeding
I = immunisation
D = skin-testing for DTH

2) We gave 200μg with each injection, whereas the strength of Husby's injection was 100μg

3) All feeds were of 50mg in both experiments. The F* indicates the extra doses of feeding that we gave to our KLH-fed group 3

4) In all cases the skin testing was performed with a 10μg intra-dermal injection

5.3.4.2 Results of Experiments on KLH-Fed Group 3

The results from this group confirm that oral feeding induced tolerance of the DTH responses. Furthermore, this tolerance is likely to be mediated by the production of a regulatory cell since feeding alone induces a positive *in vitro* lymphocyte proliferation response and after immunisation there is a positive *in vitro* lymphocyte proliferation response that does not correlate with the DTH response (see figure 5.3).

There were however differences between the results for KLH-fed group 2 and KLH-fed group 3. Firstly, there was a significantly increased lymphocyte proliferation response after feeding the higher dose of antigen, which suggests that the more prolonged course of feeding induced the production of a greater cellular response by the gastrointestinal immune system. The cells that were detected may be of the same cell type as was induced in KLH-fed group 2 or be due to the production of a different cell type that mediates tolerance. After immunisation, there was a significant reduction in lymphocyte proliferation response in the group fed the longer course of KLH compared to KLH-fed group 2. This pattern of lymphocyte proliferation responses were thus more similar to that demonstrated by Husby and suggests that the differences between my initial experiments and those of Husby are due an increase in tolerance cause by increased length of feeding rather than due to differences in methodology. The increased tolerance may be due to an increase in the activity of tolerogenic cells produced or due to the production of an additional cell type that mediates tolerance.

The antibody responses in KLH-fed group 3 showed that anti-KLH IgG responses lie at an intermediate level between those of KLH-fed group 2 and the control group but were not significantly different from either. Anti-KLH IgA responses were reduced compared to both the control group and to KLH-fed group 2, but again the difference was not significant. There may be a trend towards reduced serum anti-KLH IgA levels by increasing the dose of feeding prior to immunisation (see figure 5.4b). This potential finding should be validated by other studies.

In summary, there is a significant reduction in *in vitro* lymphocyte proliferation in the group fed the higher dose of antigen and a non-significant reduction in both IgG and IgA responses. This pattern of results showing a consistent reduction in all limbs of the immune response would be consistent with the production of more potent mechanisms of suppression by increased feeding, either by increasing the power of the same mechanism of tolerance of by the production of additional tolerogenic mechanisms. Potential additional tolerogenic mechanisms that could be induced include additional immunoregulatory cells or the induction of clonal anergy or deletion.

5.4 Hypotheses on the Possible Nature of the Regulatory Cells Induced by Feeding

It is possible to hypothesise about the nature of the putative immunoregulatory cell induced by antigen feeding by comparing the type of immune response to that seen in animal models reported in the literature (see chapter 2).

One possible cell type that can be induced by feeding animals is the T_H2 cell. This cell type, characterised in most detail in the mouse but also likely to be present in the human, provides help for humoral responses but inhibits T_H1, and hence DTH responses[62]. Subjects in KLH-fed group 2 in my experiments demonstrated a primed anti-KLH IgG response but an inhibited DTH response and therefore it is attractive to hypothesise that the production of a T_H2 response is responsible for tolerance in this group.

The observation that IgA responses to KLH, unlike IgG, are not primed would be an unexpected finding if the production of tolerance were caused by T_H2 cells. T_H2 cells, via the secretion of IL-4 and IL-5, provide help for humoral and particularly IgA responses[60]. One may also expect antigen encountered at a mucosal surface to produce an IgA response. However my results, which failed to demonstrate a priming of serum IgA responses, are confirmed by the findings of Husby *et al*[9]. There was no increase in anti-KLH IgA at the end of their immunisation schedule, although they did observe a transient increase in IgA responses after a solitary KLH immunisation. Furthermore, IgE responses, which are

strongly associated with T_H2 responses, are frequently reduced by oral exposure to antigen[15]. IgE is the antibody that is most frequently associated with food intolerance. Therefore, even in animal models in which clonal deviation has been postulated to be responsible for oral tolerance, the features of the immune response are not always typical of a T_H2 response.

There are two potential explanations of these findings. Firstly there may indeed be a production of a T_H2 response to ingested antigen, but that local factors in the gastrointestinal immune system cause it to be manifested in an atypical way. For example, Husby's results demonstrated an increase in secretary anti-KLH IgA after feeding[9]. Therefore there may be local factors that cause any IgA produced to be secreted in the gut lumen rather that into the blood stream, and thus explain why there is no increase in serum IgA. Similar local mechanisms may exist to prevent the product of IgE and hence protect against food intolerance. The other explanation is that different cell types are produced in response to oral feeding.

Another potential candidate may be the T_R1 cell. This cell type produces large amounts of IL-10, and has been shown to down regulate T_H1 responses, and lesser amounts of IL-5[139], which may conceivably prime the humoral IgG response. Thus this is a second cell type that may be responsible for the effects on the immune system causes by KLH feeding schedule 1.

The immune response to systemic immunisation in KLH-fed group 3 was similar to that seen in KLH-fed group 2 in many, but not all respects. In particular there was a significantly greater *in vitro* lymphocyte proliferation response after the feeding regime but a significantly reduced response after immunisation compared to KLH-fed group 2. There are two potential explanations for these changes. Firstly, the regulatory cell induced is the same in both groups, but that more prolonged feeding allows it effects to become more marked, possibly through stimulating the production of more of these cells or by inducing further differentiation of these cells into a more potent regulatory cell. The second explanation is that addition mechanisms of tolerance are induced. I will discuss each of these possibilities in turn.

One may postulate that the production of a T_H2 response is responsible for tolerance in both groups. T_H2 cells can inhibit the production of T_H1 responses and if T cells are induced in an environment with T_H2 cytokines present they are more likely to differentiate down the T_H2 pathway[69]. Therefore one may expect to develop more complete tolerance to T_H1 mediated immune responses such as DTH with more prolonged feeding if this were the mechanism. However, one would also expect that the T_H2 mediated immune responses, such as antibody production, would be increased by more prolonged feeding. Furthermore, if there are more T_H2 cells in the systemic circulation prior to immunisation, as is suggested by the lymphocyte proliferation assay results after feeding, one may expect the post-immunisation lymphocyte proliferation responses to be greater after immunisation in the group fed the higher dose of antigen. Neither of these findings were observed which suggests that the production of a T_H2 response is unlikely to be the cause of oral tolerance in both groups.

The production of T_R1 cells by oral feeding could be responsible for tolerance in both groups. This is an attractive hypothesis for several reasons. Firstly T_R1 cells can inhibit T_H1 functions[139] and therefore may inhibit DTH responses. Secondly it is conceivable that T_R1 cells may stimulate IgG responses through the expression of IL-5. Lastly, T_R1 cells proliferate poorly in response to antigen[139], which may explain the reduced lymphocyte proliferation seen in KLH-fed group 3. Thus it is conceivable that low dose feeding induces the production of a T_R1 cell and that the more prolonged feeding results in increased numbers of, or an increased differentiation in, these cells that alter the balance of the immune response further towards tolerance. The explanation for why there is no further increase in the IgG response may be that increased differentiation of the T_R1 cells results in reduced production of IL-5 and increased production in IL-10.

Lastly, it was observed that all aspects of the immune response measured were reduced in KLH-fed group 3 compared to KLH-fed group 2, although not all were significantly so. The trend of all these immune indicators to fall does raise the possibility that more powerful, suppressor cells are induced to mediate tolerance. One possibility is that a T_H3 type cell was

induced by feeding. This cell secretes high quantities of TGF-β which can inhibit both T_H1 and T_H2 functions[133]. The production of this cell type would be consistent with the immune response observed in KLH-fed group 3, although it would not explain the results in KLH-fed group 2 so readily. Thus if T_H3 cells are induced in KLH-fed group 3, one has to hypothesise that more prolonged feeding results in the production of addition mechanisms of tolerance in addition to those responsible for tolerance in KLH-fed group 2, and which are subsequently inhibited by the T_H3 cells. Alternatively the T_H3 cells may differentiate into its mature phenotype via stages that induce T_H2 type responses.

Finally other suppressor cells have been identified in animal models including CD8+ cells[110;111] and γδ cells[118;119]. It is possible that cells of either phenotype may be induced in humans in response to the more prolonged course of feeding and mediate the immune responses observed in KLH-fed group 3.

5.5 Summary of Experiments Using KLH to Induce Tolerance

The following conclusions can be drawn from these experiments. From KLH-fed group 1, it appears that there is a minimal dose of feeding that is required to induce detectable tolerance. The results from KLH-fed group 2 show that tolerance to DTH can occur in humans and is associated with priming of humoral responses, a pattern of immune response that may give most benefit to the host. The detection of cell proliferation in the lymphocyte proliferation assay after feeding alone suggests that tolerance is mediated through a regulatory cell, and the pattern of the immune response raises the possibility that this cell may be a T_H2 cell or a T_R1 cell. Volunteers in KLH-fed group 3 showed a reduction in *in vitro* lymphocyte proliferation after immunisation compared to KLH-fed group 2. There is also the suggestion that both IgG and IgA responses were lower in the group fed the higher dose regime of KLH and these areas should be addressed in further trials. These changes imply that more prolonged feeding either induces additional mechanisms of suppression, or that greater

numbers, or more terminally differentiated, regulatory cells are induced by more prolonged feeding which further alters the balance of the immune response towards tolerance. Work that I performed to test these theories is described in chapter 7.

6 RESULTS OF EXPERIMENTS INVESTIGATING ORAL TOLERANCE TO OVALBUMIN

6.1 Introduction

Animal studies suggest that there are multiple mechanisms of oral tolerance production. Low dose tolerance appears to be mediated by the production of active regulatory or suppressor cells, whereas high dose tolerance may be mediated through clonal anergy or deletion. The results obtained from the experiments with KLH strongly support the idea that low dose feeding in humans induces regulatory cells that mediate tolerance. This chapter examines the results of experiments performed to investigate whether high dose tolerance may be mediated by other mechanisms in humans. The method chosen to investigate high dose tolerance was to investigate the immune response to OVA, which is a common dietary antigen that will have been ingested over prolonged periods of time in the normal diet. Therefore any immunological tolerance to this antigen will be the result of "high dose" oral tolerance.

6.2 Protocol

The protocol was designed along the lines of that used for the KLH experiments. The timings of the immunisations were identical to those that produced a good immune response in the KLH control group. Two doses of immunisation were used. The first, low dose used the same dose of OVA as that of KLH (i.e. two immunisations with 200μg/ml). The second course gave 7.5 times the dose (i.e. two immunisations with 1500μg/ml). The measures used to assess immune response included ELISA measurement of anti-OVA IgG and IgA antibodies, *in vitro* lymphocyte proliferation and DTH responses. The DTH responses were assessed on consecutive days by the presence of absence of induration and the mean diameter of erythema. On the first day, a dose of 10μg of OVA in 0.1ml of saline was given. If there was no detectable erythema, a larger dose of 100μg of OVA in 0.1ml of saline was given. Readings of DTH responses were taken at 24 and 48 hours. The immunisation schedule and timing of venesections are shown in table 6.1.

Table 6.1 – Protocol for demonstrating tolerance with OVA. The timing of and doses used in the immunisation schedule are shown. Also shown are the timings at which the assays to assess the immune response were performed.

Day	Group 1 – Low dose immunisation	Group 2 High dose immunisation	Assays performed
Before trial	Normal dietary intake of OVA	Normal dietary intake of OVA	
1	Sub-cutaneous OVA (200μg in 1ml)	Sub-cutaneous OVA (1500μg in 1ml)	Lymphocyte Proliferation ELISA
11	Sub-cutaneous OVA (200μg in 1ml)	Sub-cutaneous OVA (1500μg in 1ml)	ELISA
22	Intra-dermal OVA (10μg in 0.1ml)	Intra-dermal OVA (10μg in 0.1ml)	Lymphocyte Proliferation ELISA
23	Intra-dermal OVA (100μg in 0.1ml)	Intra-dermal OVA (100μg in 0.1ml)	
23, 24 and 25			Skin tests read for induration and erythema

6.3 Results

6.3.1 Delayed-Type Hypersensitivity Testing

The skin-test results are shown in table 6.2. None of the volunteers had a positive skin test as defined by any detectable induration at the site of intra-dermal injection to either strength of injections at 24 or 48 hours. Furthermore, there was no detectable erythema in any of the volunteers to either of the doses of intra-dermal injection. Thus there was no detectable DTH response to OVA.

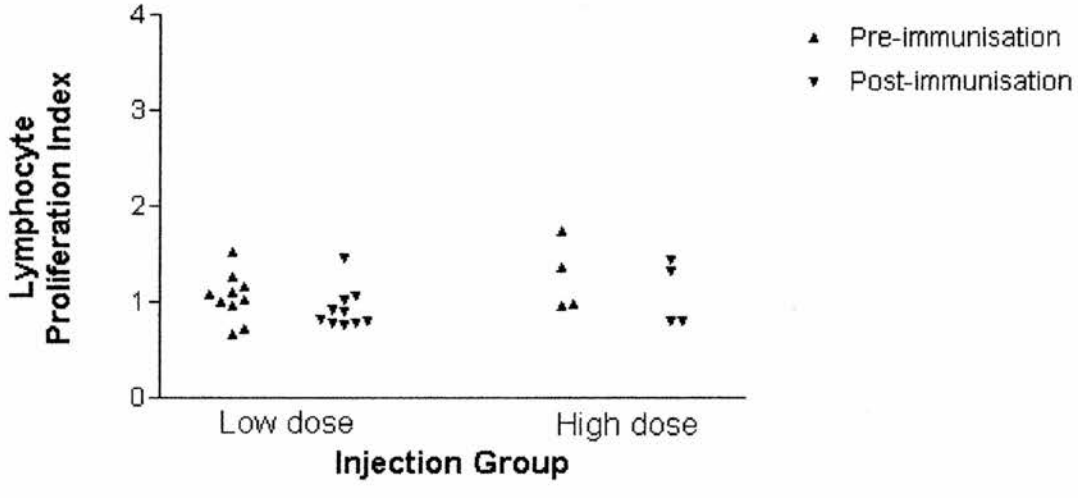
Table 6.2- Skin test results to subcutaneous OVA immunisation. The results are expressed as the number of positive results over the total number of people in that group. There was no detectable DTH response in any of the volunteers given either immunisation schedule or either intra-dermal immunisation test dose.

		Immunisation schedule	
Dose of Intra-Dermal Injection	Time of reading	Low Dose (Group 1)	High Dose (Group 2)
10μg injection	24 hours	0/10	0/4
	48 hours	0/10	0/4
100μg injection	24 hours	0/10	0/4
	48 hours	0/10	0/4

6.3.2 Lymphocyte Proliferation Responses

Figure 6.1 illustrates the *in vitro* lymphocyte proliferation responses before and after immunisation for both the group given the low dose and the high dose immunisation. There was no detectable *in vitro* lymphocyte proliferation to OVA either at the start of the experiment, i.e. after prolonged oral ingestion of OVA, or at the end of the experiment after immunisation with OVA. The results illustrated in figure 6.1 were obtained with cells stimulated with 100µl of OVA at 10µg/ml. There was no detectable lymphocyte proliferation when OVA was used at a concentration of 100µg/ml or when it was used in the assay coated to Dynabeads (data not shown).

Figure 6.1 – *In vitro* lymphocyte proliferation responses to OVA. There is no detectable lymphocyte proliferation response to OVA either after feeding of OVA or after immunisation with either of the immunisation schedules used.



6.3.3 Humoral Response

The anti-OVA IgG results are shown in graphs 6.2a and 6.2b, which illustrate the group that received the low dose immunisation schedule and the high dose immunisation schedule respectively. Day 1 values indicate the effect of long term feeding without active immunisation, day 11 values the effect of the first immunisation dose and day 21 values the effect of the full immunisation regime.

Anti-OVA IgG was detectable in the serum of all patients before immunisation in low concentrations. The low dose immunisation schedule did not cause an increase in anti-OVA IgG antibody in any of the volunteers. There was an increase in anti-OVA IgG antibody in 2 of the 4 volunteers that received the high dose immunisation schedule of 56% and 73% respectively above baseline. This result was confirmed when the specimens were run on two separate ELISAs.

The anti-OVA IgA antibody results for the low and high dose immunisation schedules are shown in graphs 6.3a and 6.3b respectively. As for anti-OVA IgG, there were detectable levels of anti-OVA IgA at day 1. Low dose immunisation had no effect on the anti-OVA IgA antibody concentration. One volunteer who received the high-dose injection schedule had a 47% increase in OVA specific IgA antibody, a result that was also confirmed in two separate ELISA runs. This was a different volunteer from the two who developed an increase in anti-OVA IgG, and therefore 3 of 4 volunteers had a rise in Ig levels as a result of high dose immunisation.

Figure 6.2a – Anti-OVA IgG levels before, during and after the low dose immunisation regime.

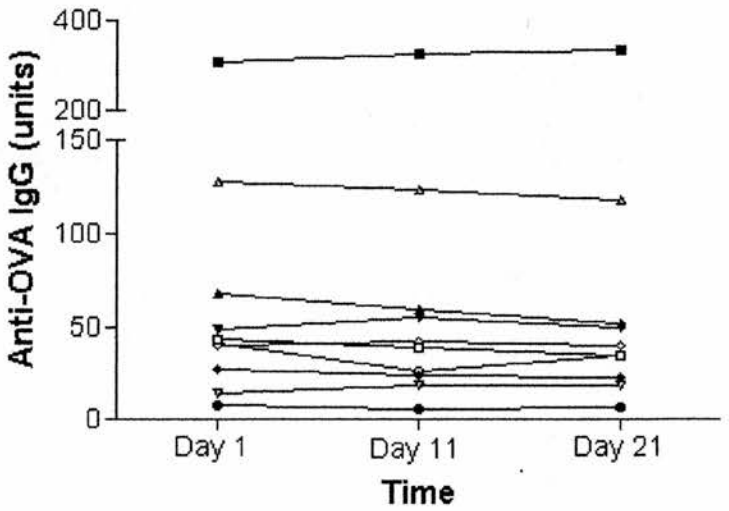
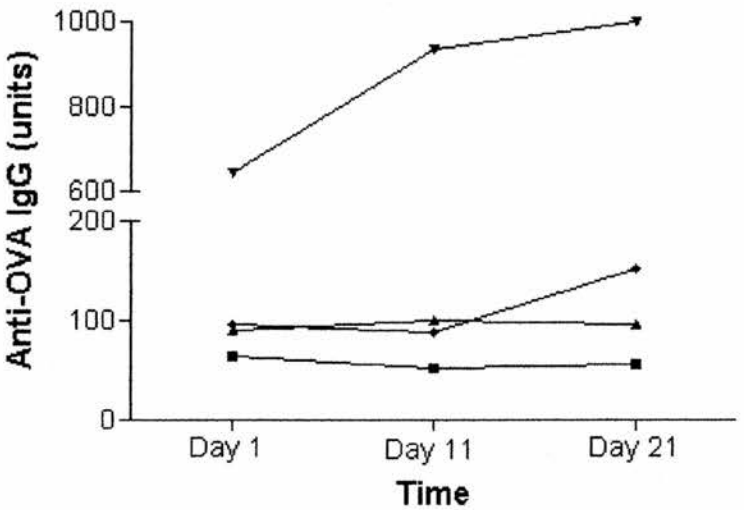


Figure 6.2b – Anti-OVA IgG levels before, during and after the high dose immunisation regime.



There are detectable levels of OVA specific IgG detectable before the immunisation schedule. There is no change in the level of anti-OVA IgG in any of the volunteers given the low dose immunisation schedule. 2 of 4 volunteers given the high dose immunisation schedule showed a small rise in anti-OVA IgG levels over baseline.

Figure 6.3a – Anti-OVA IgA levels before, during and after the low dose immunisation regime.

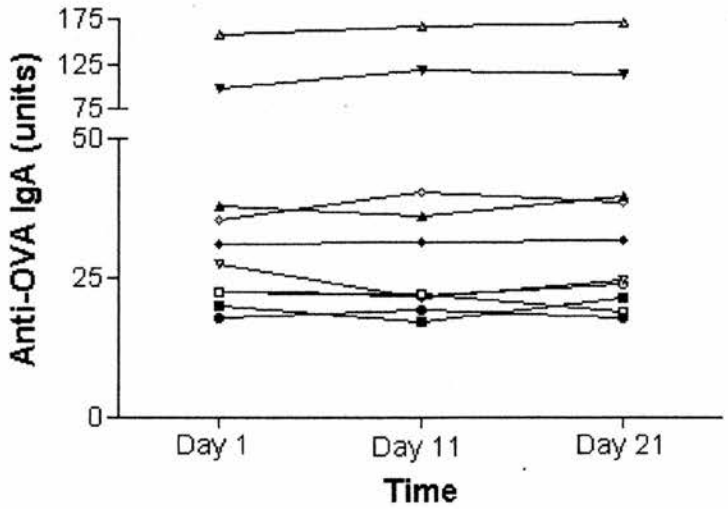
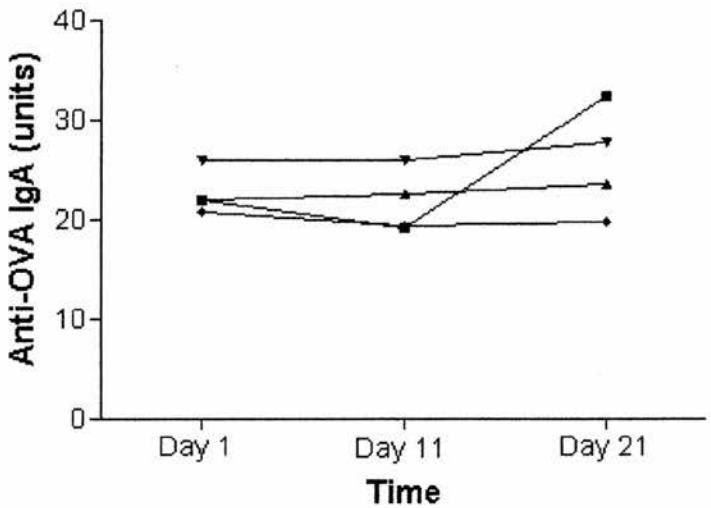


Figure 6.3b – Anti-OVA IgA levels before, during and after the high dose immunisation regime.



Anti-OVA IgA is detectable in the serum of all volunteers prior to immunisation. There is no change in quantity of anti-OVA IgA in any of the volunteers given the low dose immunisation schedule. One of the volunteers given the high dose immunisation schedule had a small rise in level of anti-OVA after the high dose immunisation regime.

6.4 Discussion of Immune Response to OVA

6.4.1 Rationale for the Use of OVA

To assess high dose feeding in humans, one has to have available an antigen that can be taken in high doses and has no toxic effects. KLH would be one possible antigen. Furthermore the high-dose feeding regime could be compared directly to the control group and those groups fed the low dose regimes. However, the cost and difficulty of obtaining KLH, associated with the onus it would place on the volunteers to take KLH for a prolonged time, made this an impractical option. The alternative approach I used to attempt to overcome these problems was to investigate the immune responses to a common dietary antigen that would thus have been encountered in high doses by all volunteers.

I chose to use OVA, which is an antigen derived from hens' eggs and is thus a common constituent in the normal diet. It is a smaller antigen than KLH, with a molecular weight of 42,700 daltons and it does not polymerise[186]. OVA was chosen for several reasons. Firstly, it has been used in the investigation of oral tolerance in many animal models in which it proved immunogenic and capable of inducing oral tolerance. Furthermore, humans produce antibodies to OVA, even in health, which suggests that it is antigenic in man. Thirdly, Sigma-Aldrich Company manufacture OVA to American Food and Drug Administration regulations and I was able to show it was sterile and endotoxin free (see Materials and Methods) and hence likely to be safe for use in humans. Lastly, the gastrointestinal laboratory has previously developed and optimised ELISAs specific for anti-OVA IgA and IgG.

6.4.2 Potential Criticisms of Experiments with OVA

There are a number of criticisms that can be levelled at these experiments. Firstly, because of the nature and choice of antigen, it was not possible to have a naïve group to act as controls. The pattern of immune response to OVA was compared to that of the KLH groups to assess

whether the two are similar or so different as to imply the operation of two different immune mechanisms. The immune response to OVA as a naïve antigen is however unknown and it cannot be assumed to mirror that of KLH. Thus there will be a speculative element to the discussion. The ideal experiments to overcome this criticism would be to give prolonged feeding schedules with KLH prior to immunisation, but funding limitations and the expense of KLH prevented me from performing such experiments.

The lack of a positive control raises the possibility that the failure to demonstrate any positive *in vitro* lymphocyte proliferation may be due to failure of the experimental technique rather than true absence of *in vitro* lymphocyte proliferation. However OVA has been used, and provoked *in vitro* lymphocyte proliferation in many animal models of tolerance. This fact and the fact that the lymphocyte proliferation assay proved reliable in the KLH experiments suggest that this was not the case. Furthermore, I used a range of OVA concentrations from 10 to 100μg/ml, in each assay to exclude the possibility that the concentration of OVA was too low. Lastly, I also used Dynabeads coated with OVA which have been shown to increase antigen uptake[187]. Failure to process and present antigen should have been overcome by these two techniques and suggest that the *in vitro* lymphocyte proliferation results may be valid.

Another methodological criticism is that the immunisation schedule given did not include adjuvant. Many animal models investigating oral tolerance to OVA used adjuvant in their immunisation schedules. Although KLH immunisation without adjuvant induced a good immune response, the same may not necessarily apply to OVA, which is a much smaller molecule. Previous work suggest that soluble antigen may not be presented *in vitro* by APCs and therefore may not cause lymphocyte proliferation[187;188]. However, soluble OVA alone can provoke egg allergy, which suggests that in some situations it can be recognised in the soluble form by the human immune system. Despite these caveats, the failure to provoke an immune response with the OVA immunisation schedule may be due to the fact that soluble

OVA is not processed by APCs and therefore is not immunogenic. Repeating these experiments with OVA plus adjuvant would be very valuable to help verify the experimental data presented here. Updated ethical approval would be required to perform these experiments.

6.4.3 The Immune Response to OVA after Oral Exposure

The volunteers who had been immunised to OVA were not naïve to OVA at the start of the experiment because all had eaten OVA as part of their normal diet over a prolonged period of time. Thus, the data on the immune response to OVA at the start of the experiment corresponded to the post eating stage of the KLH experiments.

There was a detectable, although low-level humoral response to OVA after prolonged feeding. This suggests that the gastrointestinal immune system had recognised oral OVA, and furthermore implies that in some situations soluble OVA is antigenic in humans. There was, however no detectable *in vitro* lymphocyte proliferation response after ingestion of OVA. This pattern of immune response was markedly different to that observed in the KLH experiments, where the reverse pattern of a detectable *in vitro* lymphocyte proliferation response and an undetectable humoral response was found. I postulated that tolerance to KLH was mediated by the production of a regulatory cell detectable in the lymphocyte proliferation assay. The different pattern of immune response in the OVA experiments implies that different mechanisms may be responsible for maintaining tolerance to this antigen.

6.4.4 Immune Responses to OVA after Immunisation

There was no change in either humoral or CMI after the low dose immunisation schedule. This was in contrast to the tolerance to KLH where there was a detectable *in vitro* lymphocyte proliferation response and priming of the IgG humoral response but no DTH response. The two possible explanations for the lack of response to OVA immunisation are

that there were powerful mechanisms of tolerance operating to prevent any immune response or that the immunisation schedule was not immunogenic. The best way to differentiate between these possibilities would be to give a group of volunteers OVA with adjuvant, an experiment for which we did not have ethical approval. The alternative method that I chose was to give a higher dose immunisation schedule to a trial group of four. There was no detectable CMI in any member of this group but there was a rise in antibody titre in 3 of the 4 patients. This observation suggests that the immune system did recognised OVA, but responded poorly. Although the possibility that the poor response is due to the poor antigenicity of OVA cannot be discounted, this explanation is less likely because there was a detectable immune response prior to immunisation and one would expect to a more vigorous response if the initial findings were caused by an active, non-suppressed immune response. Therefore I favour the hypothesis that the failure to respond to OVA is caused by the presence of powerful mechanisms maintaining tolerance.

The two mechanisms of tolerance identified from animal studies that would result in such non-responsiveness to OVA immunisation are clonal anergy and clonal deletion. The complete absence of lymphocyte proliferation and DTH responses to OVA are consistent with either clonal deletion or clonal anergy. The fact that there was a low level of anti-OVA antibody present suggests that deletion of B cells had not occurred. Furthermore these B cells continued to produce low levels of antibody in response to ongoing exposure to antigen as shown by the observation that antibody levels to food proteins become undetectable if that food is removed from the diet[189]. In addition I have shown that in some people, these B cells can respond to systemic challenge with high dose OVA. These facts suggest that the humoral immune system can respond to OVA, and therefore clonal deletion is not responsible for this tolerance.

The humoral response to OVA was very sluggish. For B cells to respond to antigen, co-stimulation is required from both the antigen and from help which is normally provided by antigen specific T cells[48]. Thus this anergy of the humoral response could be due to clonal

anergy of the B cells themselves or due to lack of T cell help for the humoral responses. Animal studies suggest that clonal anergy affects T cells more completely and for longer than it affects B cells[17].

The results of these experiments are consistent with the hypothesis that tolerance to OVA is the result of clonal anergy affecting OVA-specific T-cells. High dose immunisation may partially overcome this anergy by stimulating these T cells to provide some help for B cell proliferation probably through the secretion of cytokines. It may seem paradoxical that T cells can provide help for B cells without proliferating in the *in vitro* lymphocyte proliferation assay but there is evidence that anergic T cells can secrete cytokines without proliferating in other circumstances[190] and perhaps this is also occurring here.

6.4.5 Comparisons with Other Studies Investigating Immune Responses to Dietary Antigens in Humans

There are two reports of immune responses to dietary antigens that are worthy of discussion. Firstly, Brandtzaeg's group observed that antibody levels to food antigens fell with increasing age[161]. There was a wide variation of antibody levels within each age group, but overall there was a reduction that was statistically significant. One can postulate that the fall in antibody production is due to an increased total dose of antigen feeding associated with increasing age, leading to increasing anergy and/or deletion of reactive T cells

The second experiments are those of Korenblat *et al*[19]. They also injected volunteers with a food antigen, namely BSA. They observed that volunteers fell into either a group with high initial levels of anti-BSA antibody. In this group immunisation resulted in an increase in anti-BSA antibodies. The second group had low or undetectable levels of antibody to BSA and there was no increase in these antibody levels in response to immunisation. Although no comment is made on the age of each group in their paper, it is possible that older people, or those that had eaten large quantities of antigen, would have marked clonal anergy and therefore fall into the latter group. Those who were younger or had eaten less BSA would not

have developed such profound tolerance and therefore have higher initial antibody levels and be able to respond modestly to the immunisation schedule and therefore make up the first group. The results of these studies are compatible with those that I have reported.

6.5 Conclusions of OVA Experiments

In conclusion, there was an absence of CMI to OVA, both after ingestion of OVA and after subsequent immunisation with OVA. Humoral responses showed a low level of anti-OVA IgA and IgG antibodies after ingestion of OVA, but with no or very a sluggish rise after immunisation. The pattern of immune response was very different to that seen in those volunteers fed KLH, suggesting that different, more powerful mechanisms of tolerance were responsible for maintaining tolerance to OVA. This is consistent with other animal studies which show that more prolonged exposure to oral antigen leads to greater tolerance. Clonal deletion or clonal anergy are two possible candidate mechanisms.

There are criticisms that can be leveled at this work, and the findings should be confirmed by further experiments. One (expensive) way to confirm these observations would be to feed a group of volunteers regular neoantigen, such as KLH, over many months and make serial measurements of the immune responses to that antigen. This experiment would allow one to investigate how the immune responses change with increasing length of feeding. It would also allow one to see at which stage oral antigen alone produces a systemic humoral immune response and when or if this response starts to fall despite continued feeding.

7 DEVELOPMENT OF EXPERIMENTAL PROTOCOLS TO TEST HYPOTHESES OF THE NATURE OF ORAL TOLERANCE IN HUMANS

7.1 Introduction

In this chapter, I attempt to define the mechanisms responsible for the different patterns of immune responses demonstrated in patients fed KLH and OVA. The key observations that require explanation are:

1) the suppression of DTH skin tests in subjects fed a neoantigen while the lymphocyte proliferation responses and antibody production remained detectable.
2) The almost complete suppression of DTH skin tests, lymphocyte proliferation responses and antibody production to a dietary antigen fed over a lifetime.

Animal models suggests that clonal deviation (i.e. the switch from a T_H1 to a T_H2 mediated response), production of regulatory cells, clonal anergy and clonal deletion are all mechanisms mediating oral tolerance in different experimental conditions. The following experiments were designed to attempt to differentiate which of these mechanisms may be responsible for the oral tolerance induced by KLH and OVA in normal human subjects.

7.2 Experiments to Assess Clonal Deviation in KLH-Fed Group 2

7.2.1 Summary of Immune Response in KLH-Fed Group 2

Feeding volunteers 50mg of KLH for 10 days before challenging the systemic immune system with an immunisation schedule of KLH resulted in the reduction of DTH, unchanged *in vitro* lymphocyte proliferation and priming of anti-KLH IgG (but not IgA) response compared to a control group that received only the immunisation schedule. The pattern of immune response would be consistent with the postulate that feeding induced clonal deviation from a T_H1 to a T_H2 type immune response, and that these T_H2 cells mediated the observed changes in the immune response.

T_H1 and T_H2 cells are morphologically and phenotypically identical, but secrete different cytokines which defines their different functional characteristics[57]. Therefore, to investigate the hypothesis that clonal deviation is responsible for tolerance in KLH-fed group 2, I designed experiments to check possible differences in the cytokine profile of the cells taken from the groups fed KLH compared to controls.

7.2.2 Cytokine Production by Lymphocytes Measured by ELISA

The cytokine profile permits identification of the T helper cell subset. T_H1 cells produce IL-2 and IFN-γ, whereas T_H2 cells produce IL-4, 5 and 10[57]. I chose to measure the levels of IL-4 as a T_H2 marker and IFN-γ to identify T_H1 cell activity in the cell culture supernatant of cells stimulated with KLH after 24 and 90 hours of culture. These time points were chosen because IL-4 production peaks at 24 hours and IFN-γ production peaks at 48-96 hours. The samples were tested using a commercially available ELISA kit. Cell culture supernatants were collected from the cell cultures of the control group before and after immunisation and from those in KLH-fed group 2 before and after feeding and after immunisation. I postulated that if clonal deviation was caused by feeding, there should be an increase in IL-4 and decrease in IFN-γ after feeding and immunisation in the pre-fed group compared to the control group.

7.2.2.1 Results

Samples from all seven volunteers in the KLH-fed group 2 and from eight members of the control group were included. There was no detectable IL-4 in any of the specimens. Three specimens had very low levels of IFN-γ - namely one control group specimen after immunisation, one sample taken after eating KLH alone and one sample from a pre-fed volunteer after immunisation. There was no detectable IFN-γ in any other sample. Thus there

was no consistent pattern and all the positive samples gave values at the lower end of, or even just below, the lowest standard sample.

7.2.2.2 Discussion

A positive control was provided for both IFN-γ and IL-4. Both these control samples gave positive results which suggests that the assay did work in my hands. Thus the disappointing lack of results suggests that neither of these cytokines are produced or that they are produced in insufficient quantities to be detected in this assay. Husby also tried to investigate cytokine levels using cell culture supernatants[9]. In their experiments they used cytokine dependent cell-lines to measure cytokines, but they were also were unable to demonstrate the presence of any cytokines. IFN-γ and IL-4 can be difficult to detect and therefore the assays used by both Husby's group and me may not have been sensitive enough to detect the physiological quantities of cytokine produced in cell culture.

In summary, no conclusions on the nature of the tolerance in KLH-fed group 2 can be inferred from these experiments

7.2.3 IgG Subclass Levels

Another possible method to show whether clonal deviation from a T_H1 to T_H2 based immune response has been induced by feeding is to detect differences in antibody response produced. T_H1 and T_H2 cells both, to varying degrees, provide help to B cells. In animal models, the type of humoral response produced, and in particular the Ig subclasses that are produced, vary depending on the type of T cell help provided. In the mouse, T_H1 cells stimulate an IgG2a response, mediated by IFN-γ. T_H2 cells stimulate an IgG1 response mediated by IL-4 and IL-5[80]. I therefore postulated that if clonal deviation was responsible for oral tolerance to low dose feeding, there might be differences in IgG isotype production between the fed groups and the control group.

In humans, the relationship between T helper cell subset and IgG isotype production is not well established. Furthermore, the isotypes of IgG vary between mice and humans. Therefore it is impossible to draw direct parallels between any results in my experiments and those in the mouse. However, any differences in the IgG isotype produced in the different groups would imply that there are different mechanisms controlling the immune response and provide some indirect evidence for clonal deviation.

7.2.3.1 Results

The IgG1 levels after injection are shown in figure 7.1a and the IgG2 post-injection levels are shown in figure 7.1b. Although I performed this experiment to look primarily at KLH-fed group 2, the results from all the groups will be presented and discussed here for simplicity. The horizontal bars represent the median value for each group. There is no statistical difference in IgG1or IgG2 levels between any of the groups.

7.2.3.2 Discussion

These results are not statistically significant and therefore do not provide supportive evidence for the hypothesis that oral feeding induces clonal deviation towards a T_H2 cell response. There are a number of potential explanations for this result. Firstly, the numbers in each group are small, and the spread of results wide. It is therefore possible that the failure to obtain a positive result is due to type II error. Secondly there is no evidence that T_H1 and T_H2 cells cause isotype switching to different types of IgG in humans. Therefore, even if clonal deviation is induced by low dose feeding, further work on the effect of T_H2 cells on IgG isotype switching would be required to interpret this data properly. Lastly, it is possible that oral tolerance in KLH-fed group 2 is produced by a mechanism other than clonal deviation. I have hypothesised that immunoregulatory cells may be induced by feeding KLH. Other candidate immunoregulatory cells (e.g. T_R1 and T_H3 cells) on the different compartments of the immune system requires further investigation.

Figure 7-1a – Anti-KLH IgG1 levels at the end of the immunisation schedule by group

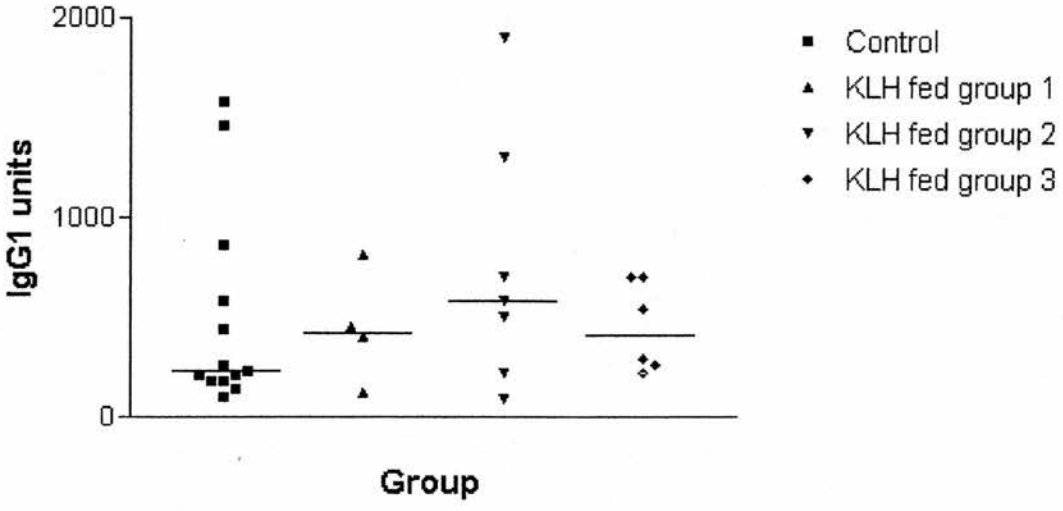
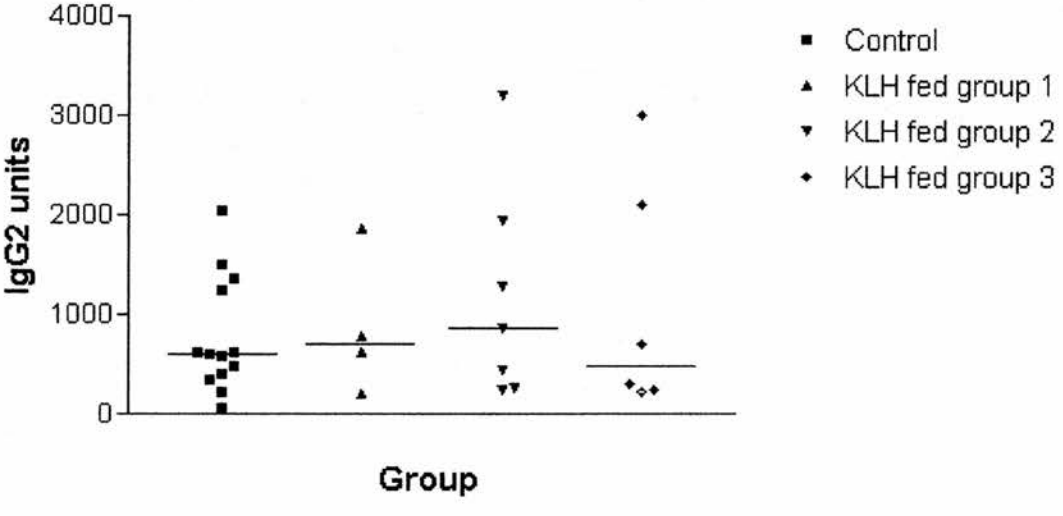


Figure 7-1b – Anti-KLH IgG2 levels at the end of the immunisation schedule by group



The graphs illustrate the values of anti-KLH IgG1 and IgG2 for each volunteer in each group at the end of the immunisation schedule. The lines represent the median value in each group. There were no statistically significant differences between any of the groups, although the range of values in each group was large.

7.2.3.4 Conclusions

In summary, the pattern of immune response observed after feeding in KLH-fed group 2 is suggestive of the induction of clonal deviation towards a T_H2 type immune response by low dose feeding. I attempted to provide supportive evidence for this hypothesis by assaying for the cytokine profile after feeding and by assessing whether the type of IgG subclass differed from the control group. I was unable to detect any significant levels of IFN-γ or IL-4 in the cell culture supernatants and there were no significant differences between the IgG subclasses produced.

There are two possible explanations for my failure to provide supportive evidence for the production of clonal deviation. Firstly, the experimental techniques may not have been able to detect clonal deviation in humans. Alternatively, it may imply that different mechanisms of tolerance are induced, such as the production of alternative regulatory cells. Further work is therefore necessary to investigate the possibility that clonal deviation can occur in humans and in parallel to assess whether alternative types of regulatory cells such as T_H3 or T_R1 cells are produced.

7.3 Evidence for Mechanisms of Tolerance in KLH-Fed Group 3

7.3.1 Summary of Immune Response in KLH-Fed Group 3

Feeding volunteers 50mg of KLH for 15 days followed by systemic immunisation resulted in an absent DTH response compared to the control group, but an unchanged *in vitro* lymphocyte proliferation response and humoral response. The *in vitro* lymphocyte proliferation response after immunisation was significantly reduced compared to that in KLH-fed group 2, and both anti-KLH IgA and IgG responses were lower, although not significantly so. These results suggest that more prolonged feeding induced more profound

suppression. This may result from either an increased production of the same regulatory cell in the KLH-fed group 2 or from the induction of an additional mechanism of tolerance.

It is unlikely that an increased T_H2 response is responsible for tolerance in both these groups since one would expect a T_H2 response to provide increased help for a humoral response and therefore increased production of antibody. It is more likely that another regulatory cell is responsible and the likely candidates that have been identified in animal studies are the so-called T_H3 cell, the T_R1 cell or an active suppressor cell. The experiments described in this section were designed to explore these possibilities.

7.3.2 Cytokine Production Measured by Cytokine mRNA Production

Cytokine production offers a powerful method of investigating the type of immune response produced and one that yielded powerful evidence of the mechanisms of tolerance production in many animal models. In the previous section, I reported failure to detect cytokines in KLH-fed group 2 using an ELISA based method, possibly because the assay was not sensitive enough. I therefore developed a PCR method to measure cytokine mRNA as a potentially more powerful assay to detect cytokine production.

PCR assays are much more sensitive that ELISA techniques and may detect even modest increases in cytokine production. However, there are several drawbacks in using these techniques. Firstly, PCR detects cytokine mRNA rather than the cytokine itself. Although the quantities of the two should correlate closely since cytokine production is transcribed from its mRNA template, this may not always be the case. Another significant problem is that it is very difficult to get accurate quantitative results. The multiple amplification stages in PCR result in even very low levels being detected and it can be difficult to compare the quantity of initial cytokine mRNA between different samples. I used a computer based analysis technique to attempt to overcome this problem, which is not a quantitative technique and the results therefore have to be interpreted with caution.

7.3.2.1 Development of the PCR Assays

The methods used were derived from those of Jarvis *et al*[182] and the primers used were the same. A series of experiments were run to optimise the concentration of magnesium and primer, the annealing temperature and the number of PCR cycles for use in our laboratory. cDNA derived from cells that had been stimulated by con A (a non-specific T cell mitogen) was used as a positive control to optimise the assays for cytokine mRNA. Attempts to multiplex the primers – i.e. run actin and cytokine PCR assays in the same tube – failed probably because of primer dimerism.

It proved difficult to develop a standard assay to detect IFN-γ and IL-10 mRNA. A hot start technique was developed with success. This technique involved adding the Taq DNA polymerase to the sample at a temperature of 80^0C. This ensures that the entire DNA is fully unravelled, and therefore the primers bind more specifically to their complimentary sequences. At lower temperatures the primers may bind more non-specifically and give false bands. The technique was also successfully applied to IL-2 and actin and was used in all experiments.

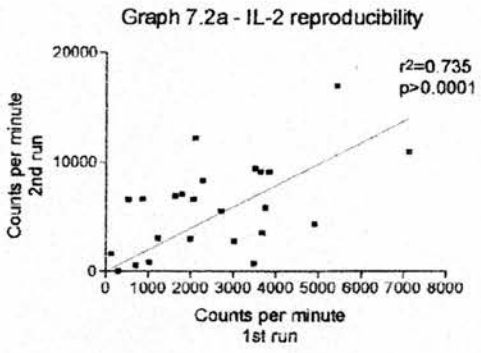
The IL-4 mRNA PCR assay proved difficult to optimise. Changes to the annealing temperature and number of PCR cycles were made in an attempt to get clear bands of the predicted size. Even with these conditions, a double band was produced and the IL-4 PCR assay proved to be the least reliable.

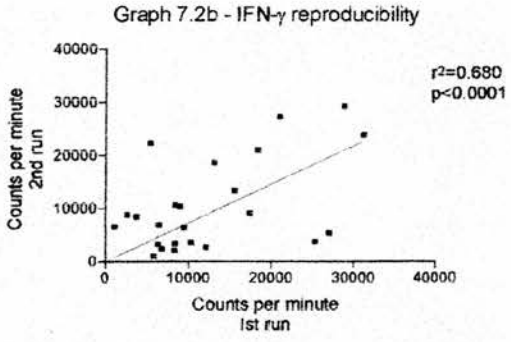
7.3.2.2 Reliability of the PCR Assays

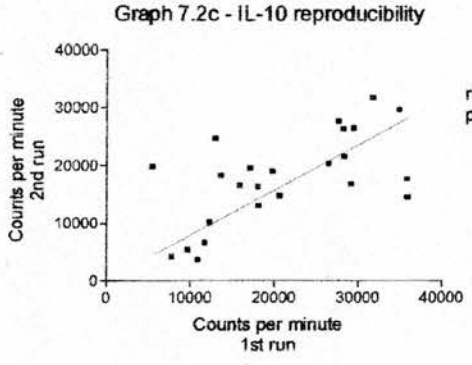
The PCR method used is not a quantative technique. I therefore had to attempt to establish that the results are valid and reproducible. A coefficient of variance could not be calculated because of the small amounts of cDNA produced from each sample. Several methods were employed to overcome this problem. Firstly, controls were used which should give high values. The fact that high levels were obtained in these samples, as will be discussed below,

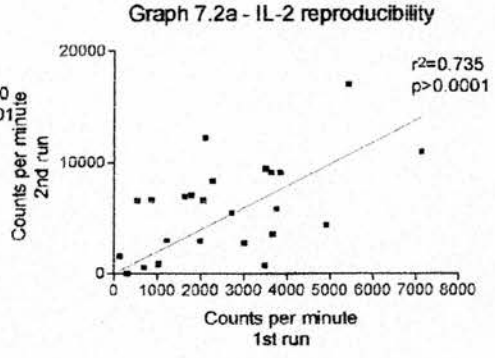
is encouraging and suggests that the assay can detect samples containing high amounts of mRNA. Secondly, all samples were run twice and results from each run were compared. The mean variability between the same sample run on different runs was 88% for IL-2, 71% for IFN-γ, 37% for IL-10 and 76% for IL-4. These results are shown graphically in figures 7.2a-d. Thus, the correlation between the individual values run on separate gels was sub-optimal. Part of the variability between runs may have been due to different degrees of staining with ethidium bromide on different gel. To remove this source of error, I ran and developed all the samples that I was going to compare on the same gel. Furthermore, all samples were run on two different occasions and the pattern of results seen on each occasion was similar. Therefore, the pattern of results obtained from the different groups may be a true reflection of the absolute level of mRNA in each sample.

Figures 7.2a-d – these graphs show the reproducibility of the IL-2 mRNA, IFN-γ mRNA, IL-10 mRNA and IL-4 mRNA PCR assays respectively. Each sample was run on two sperate occasions, labelled 1st rub and 2nd run. The values given are of counts per minute of fluorescence under ultra-violet light recorded by the molecular analyst program. The results for the PCR products obtained at each assay were compared by linear regression to assess reproducibiliy.









7.3.2.3 Results of Testing for Cytokine mRNA

The cytokine mRNA levels and actin levels are shown in graphs 7.3a-e. A typical gel is presented with each figure (photos 7.1a-e) to illustrate the pattern of bands obtained.

The actin results showed that a consistent level of actin mRNA was obtained from each sample. The results for IL-2 and IFN-γ showed that there was increased production of these pro-inflammatory cytokines in the control group after immunisation compared to pre-immunisation. Furthermore, stimulating cells with conA or PPD also results in an increased production of mRNA of these pro-inflammatory cytokines compared to that produced by unstimulated cells. Feeding alone did not increase the levels of these cytokines. After immunisation of the pre-fed group, there was a trend towards increased IL-2 mRNA production, although not to the level seen in the control group, whereas IFN-γ levels were not increased over baseline. There was no statistical difference between mRNA IL-4 levels in any of the groups. There was a very wide range of mRNA IL-10 levels at baseline. All the samples tested after immunisation of the control group show mRNA IL-10 levels at the upper limit of baseline levels. There was a significant reduction of IL-10 mRNA levels after feeding and after immunisation of the KLH-fed group compared to the control group after immunisation.

Pictures 7.1a – 7.1e show typical gels obtained for the actin, IL-2, IFN-γ, IL-10 and IL-4 assays respectively. On each gel, lanes 1 and 2 contain a pre- and post-immunisation sample from a volunteer in the control group. Lanes 3 and 4 are from pre- and post-immunisation samples from a second volunteer in the control group. Lanes 5, 6 and 7 contain the pre-feeding and pre- and post-immunisation samples from one volunteer from KLH-fed group 3 and lanes 8, 9 and 10 contain samples from another volunteer in this group at the same time-points. Lanes 11 and 12 contain 2 samples from 2 separate cell cultures stimulated with conA. The last lane in the gel (except on the actin gel where it is on the left) contains a sample run with no cDNA and acts as a negative control. The DNA ladder, used to calculated the size of the samples of cDNA can also be seen on each gel. The size of each rung of the DNA ladder, in base pairs are shown in the adjacent text boxes.

Figure 7.3a – 7.3e show the individual levels of actin, IL-2, IFN-γ, IL-10 and IL-4 respectively at each time point, expressed as the level of fluorescence detected by the molecular analysis program in counts per mm^3. Samples from all subjects prior to KLH exposure are included in the pre-antigen group. The next column shows the values obtained after immunisation in the control group. The samples from KLH-fed group 3 after the feeding schedule and after immunisation are shown in the next 2 columns. Finally the positive controls of samples stimulated with PPD and conA are shown.
The results illustrate that a consistent level of actin cDNA is obtained from each sample. IL-2 and IFN-γ levels are increased after immunisation in the control group ($p<0.01$ and 0.07 respectively), but IL-10 and IL-4 levels are not raised.
After feeding there is no detectable rise in any cytokine compared to baseline, and IL-2, IFN-γ and IL-10 levels are significantly less than the control group after immunisation. After immunisation of KLH-fed group 3, there is a trend towards increased IL-2, but not IFN-γ and IL-10, compared to baseline. The levels of IL-10 are significantly less than the control group after immunisation.

Picture 7.1a – Typical results of from an actin PCR experiment.

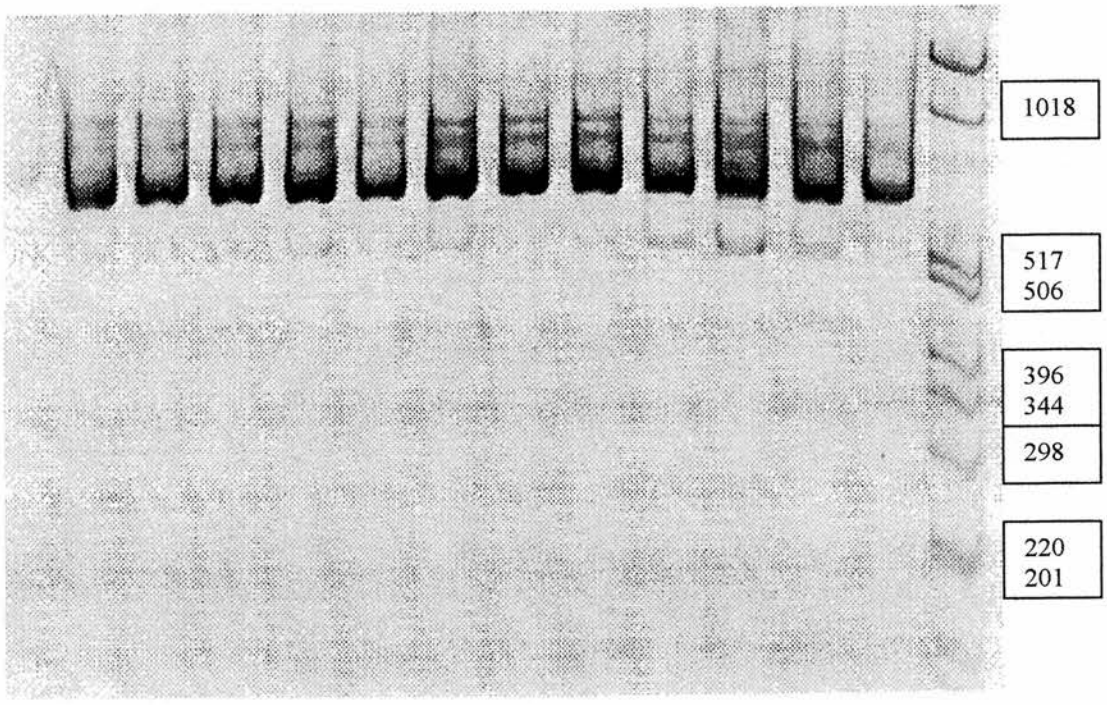
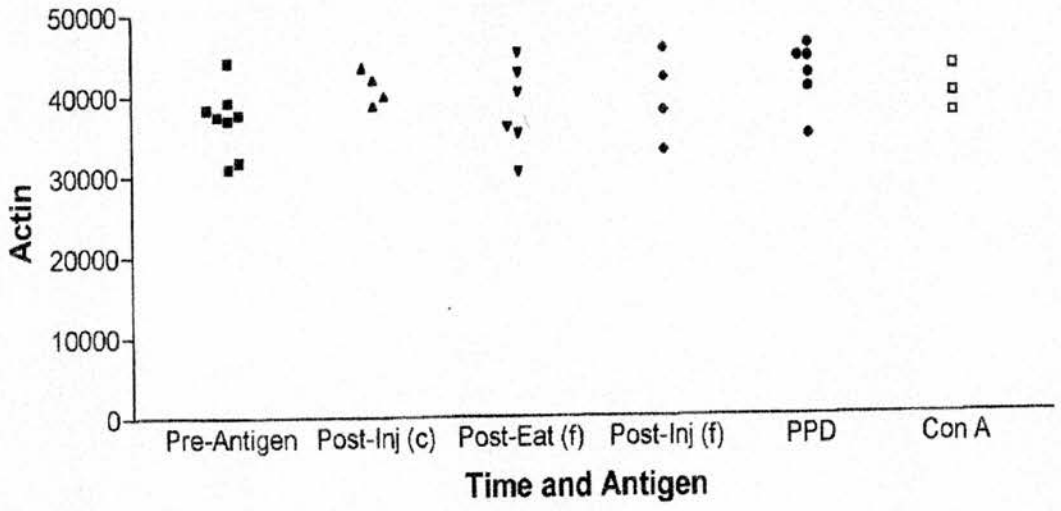


Figure 7.3a – Actin mRNA levels by group



Picture 7.1b – Typical results of from an IL-2 PCR experiment.

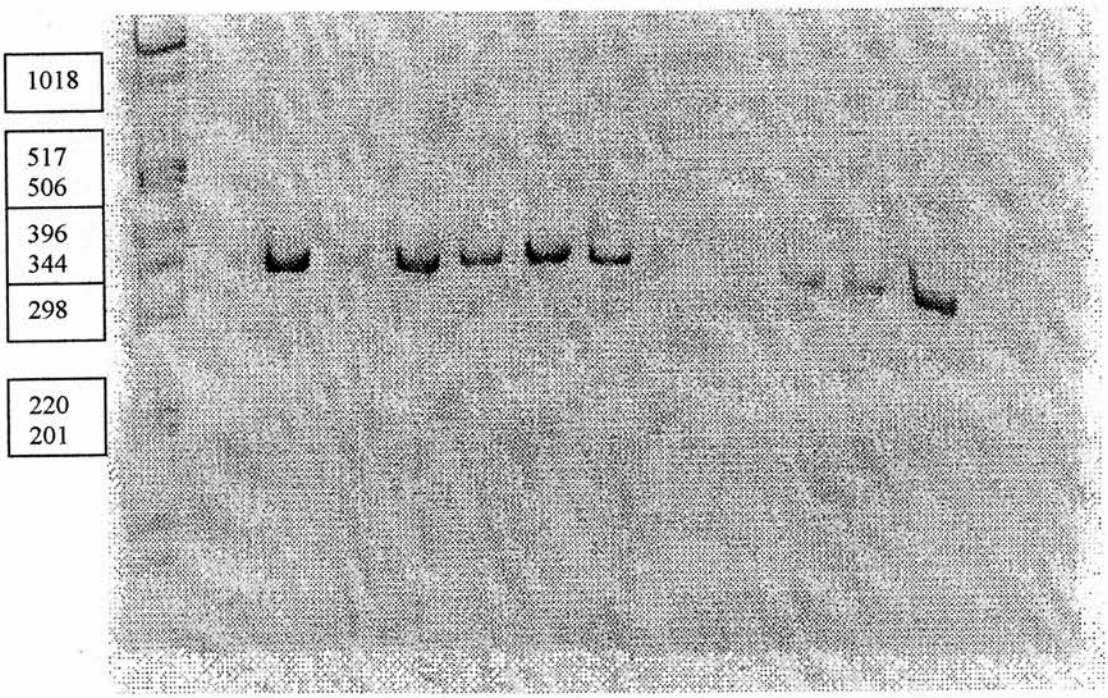
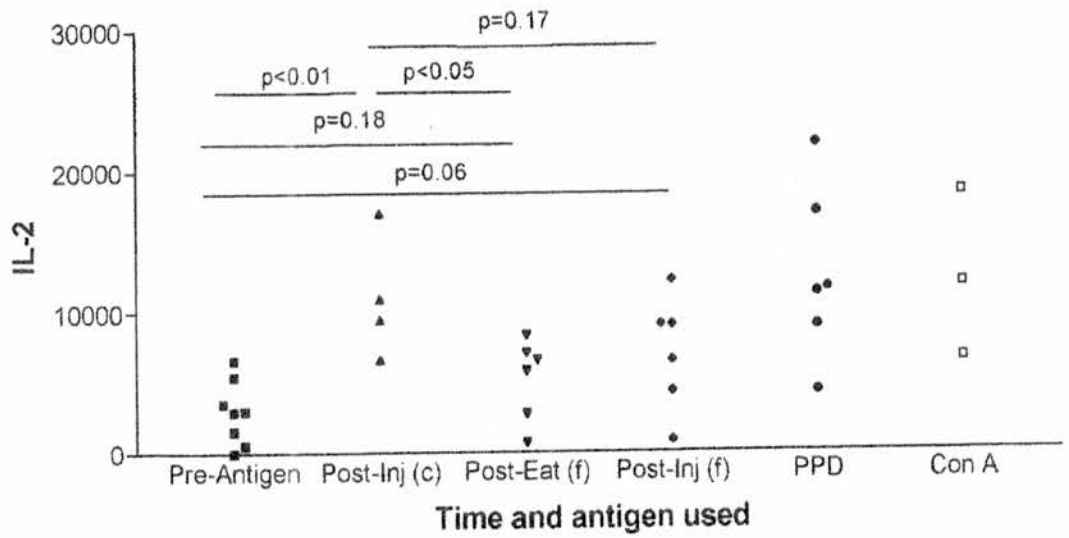


Figure 7.3b – IL-2 mRNA levels by group



Picture 7.1c – Typical results of from an IFN-γ PCR experiment.

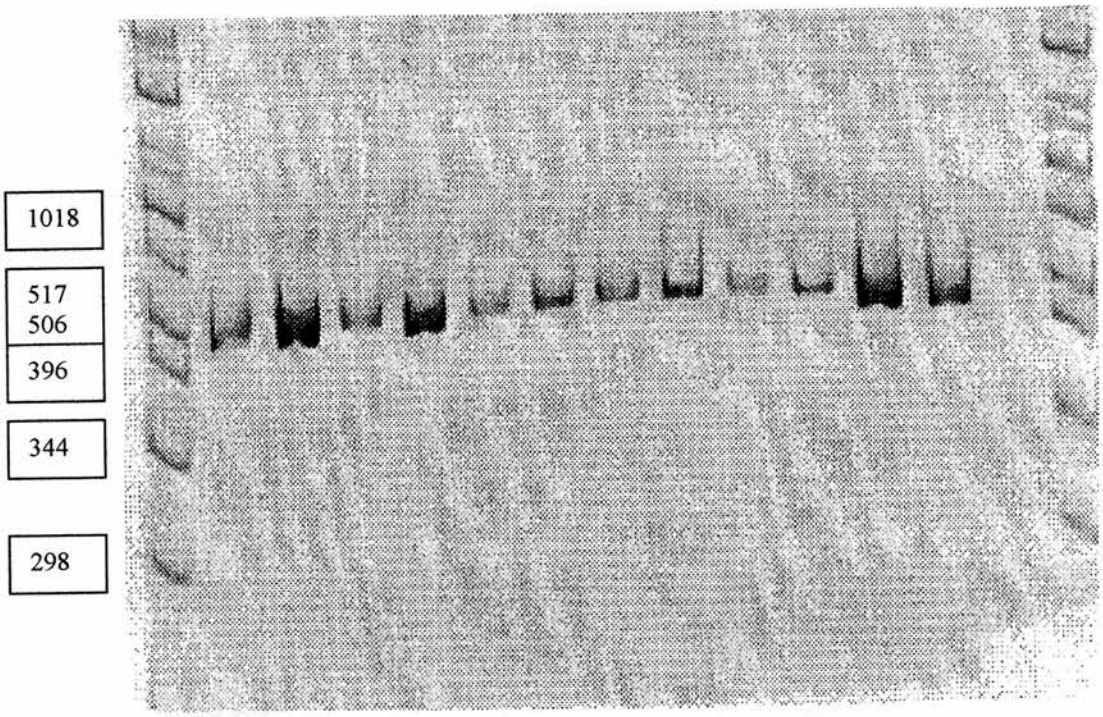
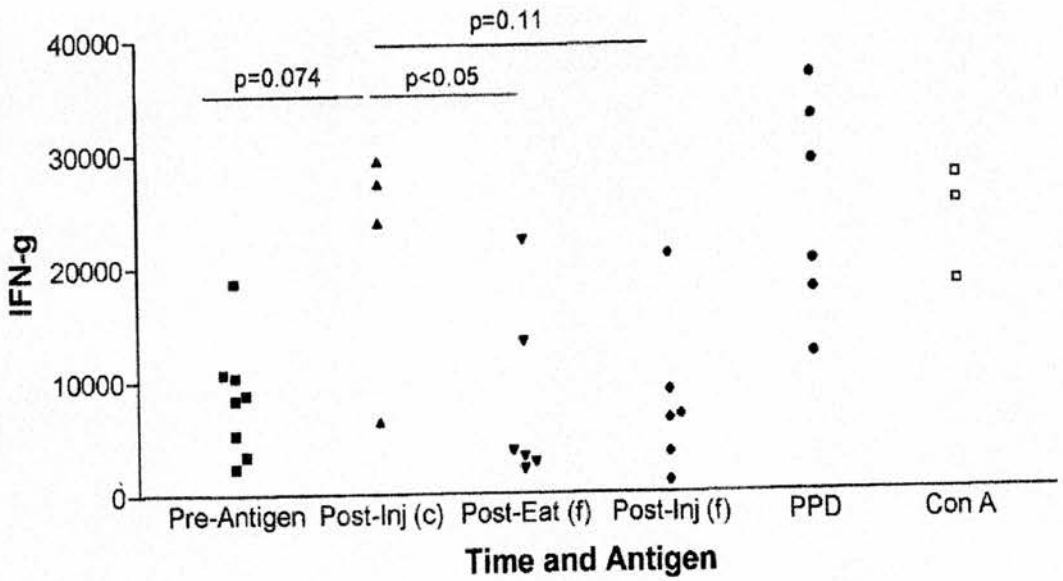


Figure 7.3c – IFN-γ mRNA levels by group



Picture 7.1d – Typical results of from an IL-10 PCR experiment.

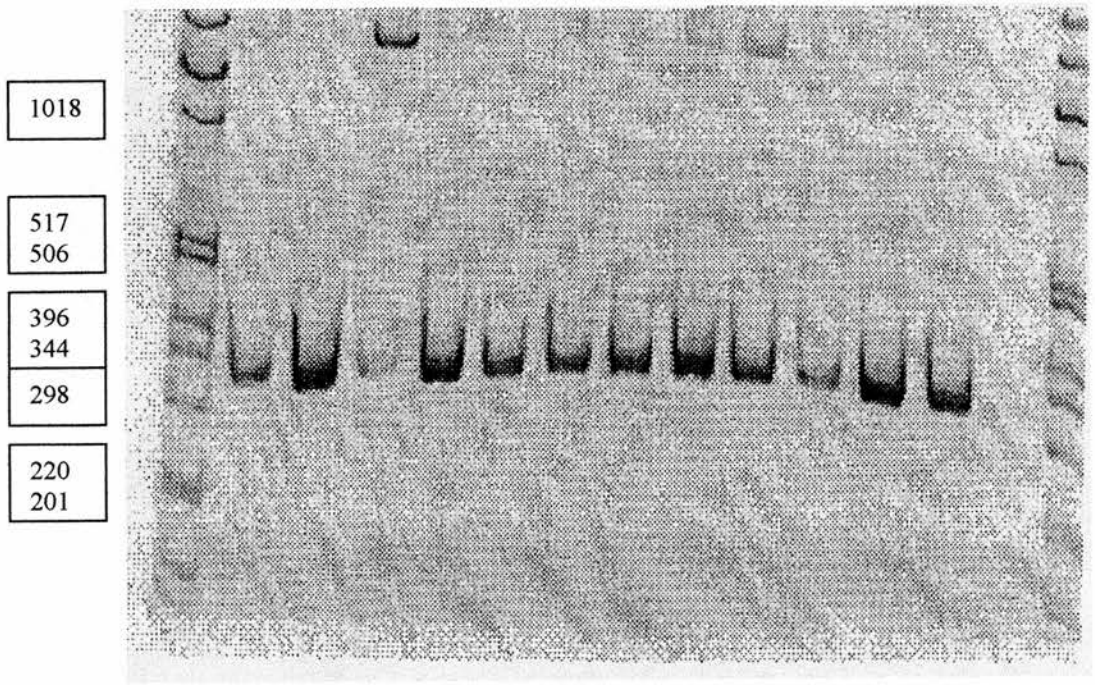
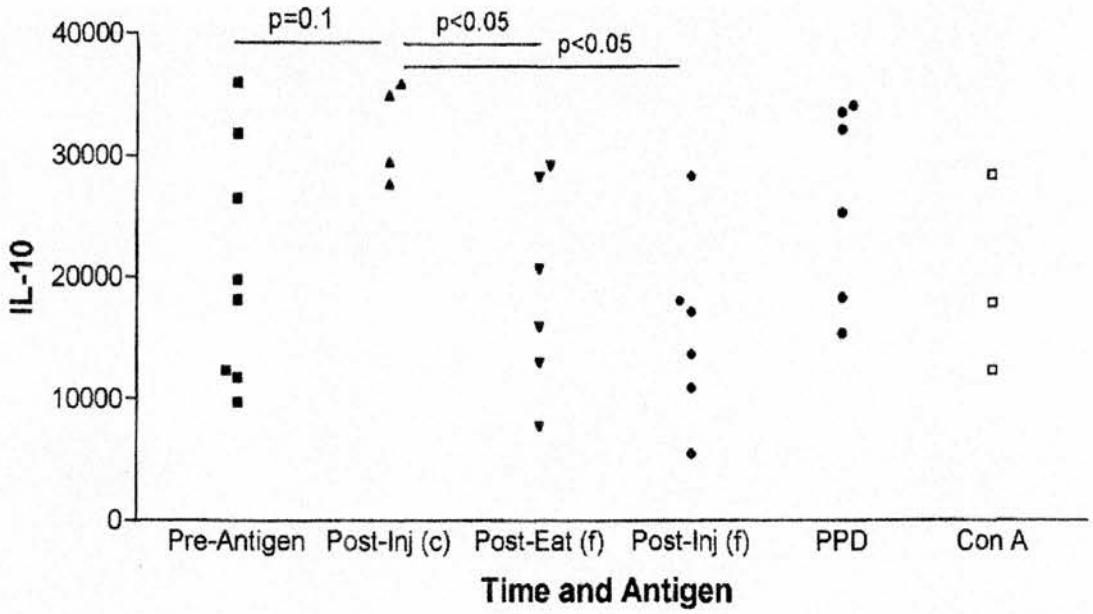


Figure 7.3d – IL-10 mRNA levels by group



Picture 7.1e – Typical results of from an IL-4 PCR experiment.

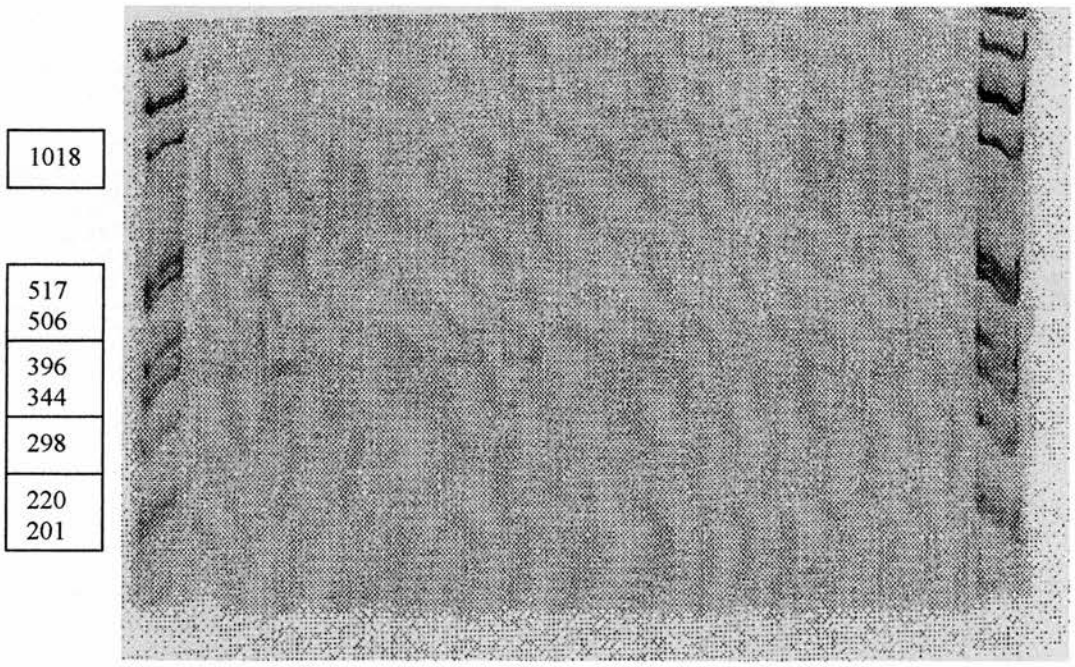
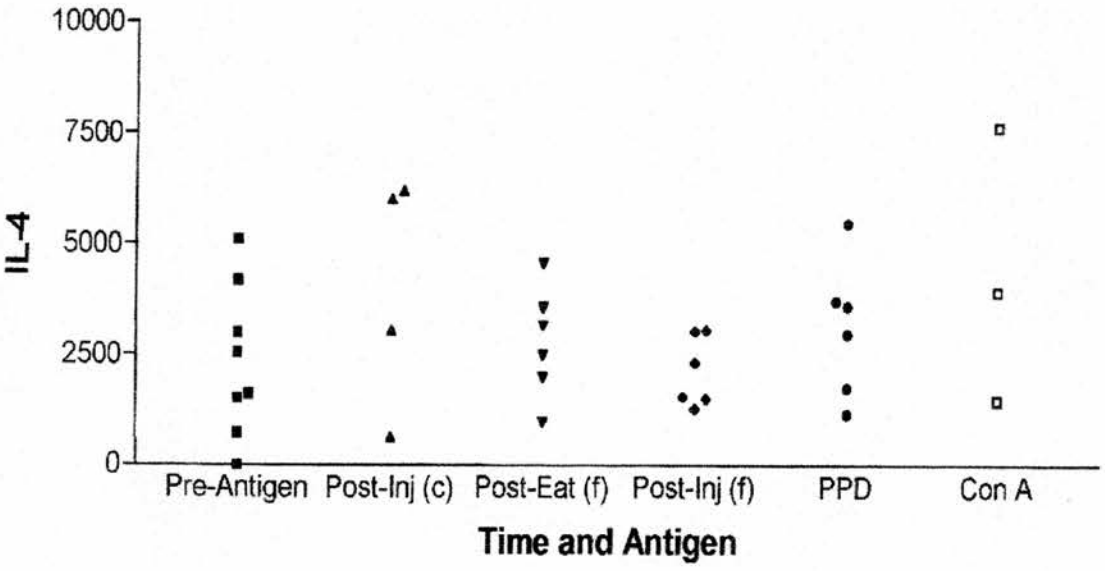


Figure 7.3e – IL- mRNA levels by group



7.3.2.4 Discussion of Cytokine mRNA Production

7.3.2.4.1 Validity of the Assays

There are a number of problems with using this technique to assay cytokine production that have been referred to in the introduction. Firstly, this technique tests for cytokine mRNA rather than the cytokine itself. Secondly this is not a quantitative PCR technique and therefore comparisons between the groups may be misleading. Lastly, the PCR technique is so sensitive that even small amounts of mRNA can be detected. This final point is illustrated by the fact that cytokine mRNA for each cytokine tested for is detected even from unstimulated cells that do not produce a response in the lymphocyte proliferation assay. Indeed, at baseline there was a wide range of cytokine mRNA values particularly for IL-10 mRNA, which in turn reduces the likelihood of obtaining statistically significant results in such a small sample size.

Some of the results do suggest that the assays may indeed give an accurate reflection of the cytokine levels. Firstly, the levels of actin mRNA from each sample was constant. Actin is a molecule important in maintaining the cell cytoskeleton and therefore should be produced in equal quantities in all groups regardless of whether the cells are stimulated by antigen or not. The presence of similar levels of actin in all groups suggests that the extraction process for mRNA worked well.

Cells stimulated with conA and with PPD were used as positive controls. ConA is a non-specific T cell mitogen and PPD has been used in the immunisation programme against tuberculosis and therefore both these antigens should activate T cells. In both cases there was a significant increase in IL-2 and IFN-γ mRNA compared to non-activated cells. These results also suggests that these assays worked well. There was no increase in IL-10 or IL-4 production in cells stimulated with either of these antigens. This is consistent with a T_H1 type response to these antigens. However, it is possible that the IL-4 and IL-10 mRNA assays are

not as reliable and the lack of a positive control suggests that the results of these two assays should be treated with caution.

Lastly all the samples were run on two separate occasions and the pattern of results was similar in each, which suggests that the assays gave reproducible results.

7.3.2.4.2 Cytokine mRNA Production in the Control Group

Immunisation with KLH caused a positive immune response detected by positive DTH skin tests, *in vitro* lymphocyte proliferation and antigen specific IgG and IgA. This was associated with the production of significantly increased amounts of IL-2 mRNA in cell culture along with a trend towards increased amounts of IFN-γ mRNA. There was no increase in IL-4 or IL-10 mRNA, although the levels of IL-10 mRNA were all in the upper range of the wide band of levels seen in the control group. These results are consistent with the production of a predominantly T_H1 immune response following immunisation with KLH and is what one would have predicted. Furthermore, the subject whose cells produced the lowest levels of IL-2 and IFN-γ mRNA to KLH had a low *in vitro* lymphocyte proliferation result of 1.8 which implies that samples that fail to proliferate in the *in vitro* proliferation assay in response to KLH do not produce large quantities of cytokine mRNA.

7.3.2.4.3 Cytokine mRNA Production in the KLH-Fed Group

After the feeding regime of KLH there is no increase in IL-2 or IFN-γ mRNA compared to baseline and a significant reduction in both compared to the control group post immunisation. Feeding did result in an increase in *in vitro* lymphocyte proliferation and I hypothesised that this represented the production of an immunoregulatory cell (discussed in chapter 5). The differences in IL-2 and IFN-γ mRNA production suggest that this is a different cell type to that induced by immunisation and would be consistent with this conclusion.

Possible candidates for this immunoregulatory cell include the T_H2 cell and the T_R1 cell. There is no increase in IL-4 or IL-10 mRNA levels over baseline. Indeed there is a significant reduction in IL-10 mRNA compared to that seen in the control group after immunisation. Both these cytokines are produced by T_H2 cells[80] and IL-10 is produced by T_R1[139]; thus these results fail to provide supportive evidence that either of these regulatory cells are responsible for low dose oral tolerance in humans. Either the assays for these cytokine mRNA levels are not reliable, a possibility that has to be considered particularly in the absence of a positive control, or a different type of regulatory cell may be responsible for tolerance. One possible candidate would be the T_H3 cell which exerts its actions via the secretion of TGF-β[133]. Clearly, this area would benefit from further work.

After immunisation of the pre-fed group, the pattern of cytokine mRNA production showed a more confused pattern. There was a trend towards increased IL-2 mRNA over baseline, but not to the level of the control group after immunisation (p=N.S.). There was no increase in IFN-γ mRNA over baseline, and a trend towards a reduced level compared to the control group post immunisation. IL-10 mRNA levels were similar to baseline and significantly reduced compared to the control group post immunisation. IL-4 levels were similar in all groups. The intermediate pattern of pro-inflammatory cytokines is difficult to interpret but may represent a balance between the pro-inflammatory response to immunisation and the inhibitory response of the immunoregulatory cells induced after feeding. Measurement of the immunoregulatory cytokines' mRNA does not provide any definitive data, but are not consistent with the production T_H2 or T_R1 cells. Further work is needed to ensure that these results are reproducible and to investigate whether other cytokines such as TGF-β may be responsible for tolerance production.

7.3.3 Bystander Suppression

7.3.3.1 Introduction and Rationale

My experiments suggest that feeding KLH to humans induced tolerance to DTH responses associated with the production of an immunoregulatory cell. The nature of this cell is unknown. In certain animal models feeding of antigen induces a suppressor cell which can be identified by both *in vivo* transfer experiments[11] and in *in vitro* lymphocyte proliferation assays[83]. These experiments suggest that suppressor cells are activated in an antigen specific manner, but act to produce their effects in an antigen non-specific manner such that they can inhibit the immune response to a second, unrelated antigen. This has been termed bystander suppression[83].

In this section I describe the results from experiments using an assay that I developed to assess bystander suppression in humans. A second unrelated antigen, PPD, to which most people mount a good immune response was added to mononuclear cells in the *in vitro* proliferation assay. Other cells were cultured with KLH and PPD in combination. A suppression index was calculated by dividing the results of cells cultured with PPD alone by that of cell cultured with KLH and PPD. Values greater than one imply that there is inhibition of the PPD response by cells activated by KLH – i.e. there is bystander suppression.

7.3.3.2 Results of Bystander Suppression Assay

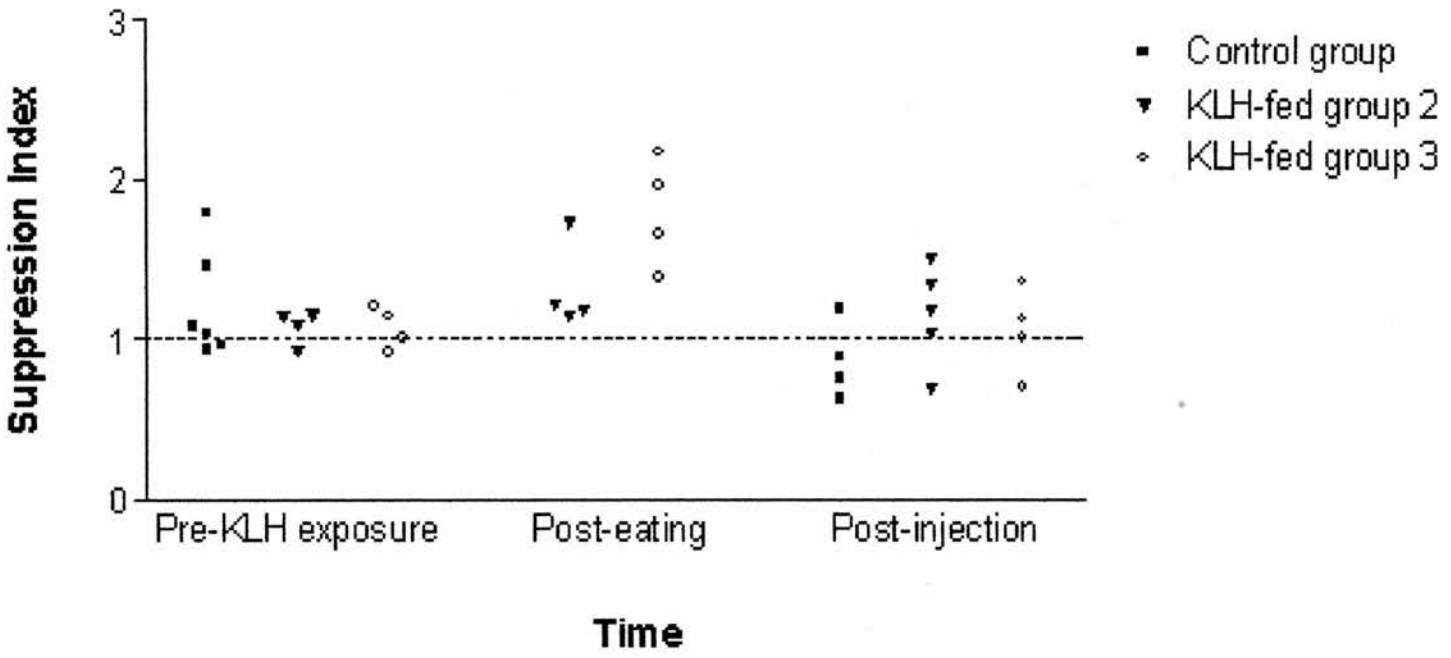
The results are shown in figure 7.4. The dotted line shows a suppression index of one which would be expected if there were no suppression. Results were obtained from four volunteers in each of the control group, KLH-fed group 2 and KLH-fed group 3. The numbers in each group were small and therefore the results should be treated with caution.

There was no evidence of suppression of the proliferation response to PPD in cells co-cultured with KLH before the subjects had had any exposure to KLH, although two values

obtained from the control group were greater than one. After immunisation of the control group, there was no evidence of bystander suppression. Indeed three of the four values were less than one.

After feeding of the lower dose of KLH, there was no evidence of bystander suppression. After feeding of the more prolonged course there was an increase in suppression ($p=0.03$ compared to pre-feeding values). Following immunisation, there was no evidence of bystander suppression in either KLH-fed group 2 or 3.

Figure 7.4 – Results of the bystander suppression assay are shown. A suppression index of one indicates no bystander suppression, whereas results greater than one indicate evidence of bystander suppression. There is no evidence of bystander suppression in any of the groups prior to exposure to KLH, or after immunisation of the control group with KLH. Feeding KLH to group 3, but not group 2, resulted in bystander suppression of the PPD response. There was no evidence of bystander suppression in either of the pre-fed groups after immunisation.



7.3.3.3 Discussion – Has Bystander Suppression Been Generated

To assess bystander suppression, an unrelated antigen that provokes an immune response is required. PPD was chosen as the second antigen because most people have been immunised with PPD and should have a good immune response against it. Furthermore, it was found that lymphocyte proliferation to PPD was better than to other antigens such as tetanus toxoid and hepatitis B surface antigen. An alternative approach would be to give an immunisation schedule of an unrelated antigen to induce (or boost) the immune response to that antigen. I did not wish to do this for two reasons – namely I felt the results would not be directly comparable to previous experiments since it is possible the two immunisation schedules may interact and, secondly, I did not wish to impose a greater burden on the volunteers.

When developing a new technique in the laboratory, one has to ask whether the assay measure what one designs it to measure. This is particularly true in an assay such as this in which no positive controls were available to optimise the assay. In this respect, the samples taken before the volunteers were exposed to KLH act as an important negative control. 12 of these 14 samples demonstrate a value around one. This suggests that the presence of KLH in the cell culture does not inhibit cell proliferation to PPD and therefore give falsely positive evidence of bystander suppression. Two of the baseline samples did give values markedly greater than one which suggests that false positive values can occur. Both samples were run at the same time. Further work suggested that one particular batch of KLH induced this response, and that there was no non-specific inhibition of the PPD response when different batches of KLH were used. The reasons for this one batch of KLH causing such a problem are unknown. Thus although non-specific inhibition of the PPD response may occur, it did not seem to be a common problem in this assay.

After immunisation of the control group, which produced an active immune response as shown in previous experiments, there was no evidence of bystander suppression. This suggests that the active immune response to KLH did not inhibit lymphocyte proliferation to PPD *in vitro* – i.e. there was not falsely positive evidence of bystander suppression caused by

two active immune responses interfering with each other in the reaction. Furthermore, in three of the four subjects the suppression index was less than one, which may imply that the two antigens caused additive lymphocyte proliferation in the assay. These results also suggest that the assay is valid.

After feeding of KLH, there was evidence of suppression in KLH-fed group 3, but not in KLH-fed group 2. In both groups, the presence of a detectable lymphocyte proliferation response after feeding and changes in the immune response to immunisation compared to the control group suggested that immunoregulatory cells were induced by feeding. This result implies that a more powerful regulatory cell was induced by more prolonged feeding, but does not differentiate between the possibilities that either an additional type of regulatory cell was induced by feeding or that more prolonged feeding resulted in the development of greater numbers or an increasingly differentiated regulatory cell that mediated the response in both groups.

Work from animal studies suggests that suppressor cells can exert their effect by secreting cytokines[83]. It would be fascinating to develop the bystander assay to see if one could identify any suppressive cytokines in humans. One could add anti-cytokine monoclonal antibody to the assay to assess whether the bystander suppression was attenuated. For example, one such cytokine that may be involved in the induction of oral tolerance is TGF-β[135]. Inhibition of bystander suppression by anti-TGF-β antibody would provide supportive evidence to the hypothesis that T_H3 cells may mediate oral tolerance in humans.

After immunisation, there was no longer any detectable bystander suppression in either of the pre-fed groups. For KLH-fed group 2, this result is in keeping with the results before immunisation and suggests that cells with suppressive properties were not induced at this dose of feeding.

Conversely, for KLH-fed group 3, the fact that suppressor cells were detected after feeding but not after immunisation is unexpected. This may suggest that the results are related to a

failure of the assay rather than a genuine finding, particularly as small numbers of volunteers are included in these experiments. These results need to be replicated elsewhere. If one assumes these results are genuine and not due to statistical error, potential hypotheses that may help to explain them include:

1) Human suppressor cells (in this section I use the term suppressor cells to described the cells that inhibited the PPD response in the lymphocyte proliferation assay, although the nature of the cell is unknown and may be a powerful regulatory cell) are active only for a short period of time and are susceptible to clonal anergy which renders them incapable of reacting in the *in vitro* bystander suppression assay.
2) The suppressor cells may be induced to undergo apoptosis or clonal deletion. This theory may be supported by the observation that *in vitro* lymphocyte proliferation is reduced after feeding the more prolonged course of KLH. However, the feeding schedule does influence the immune response to systemic immunisation, which makes this hypothesis less likely.
3) The route and method of antigen presentation is crucial in determining whether these suppressor cells are activated so that systemic presentation of antigen does not activate suppressor cells, but oral presentation does, possibly by providing the necessary co-stimulation to induce suppressor cells. However, the systemic immune response was clearly influenced by oral antigen since the DTH response to systemic immunisation was reduced.
4) Immunisation provokes an active immune response that is attenuated by suppressor cells in the circulation. Therefore, the balance between the suppressive response and the active response to KLH in the *in vitro* assay was altered by immunisation such that the unrelated response to PPD was no longer inhibited.
5) The suppressor cells that are induced in the gastrointestinal immune system by feeding may home back to GI sites and therefore not be present in the systemic circulation at the time of venesection. It is possible that these cells do influence the response to

immunisation by altering the immune response at the time of KLH immunisation, but that they are no longer present in sufficient numbers in the serum at the time of venesection to inhibit the unrelated PPD response.

The presence of bystander suppression is very important if oral tolerance is to be used as a therapeutic tool to treat autoimmune diseases. Most human autoimmune diseases are caused by an immune response to multiple antigens within the target tissue and often the antigen that initiates this response is unknown. Therefore the induction of a suppressor cell by feeding antigen from the target organ that will inhibit the immune response to unrelated antigens in that organ offers the best hope using oral tolerance to treat these diseases. These results offer some hope that this approach may be feasible. However, they were obtained on a small sample size and were only demonstrable at one time point shortly after feeding. Further work is needed to confirm these results, to identify the nature of the suppressor cell and determine why it was not detectable after immunisation. In addition, these experiments were performed by antigen given orally before systemic challenge. In autoimmune disease, the immune response is already present and further work is necessary to assess whether oral tolerance, and particularly bystander suppression, can down regulation an existing immune response.

7.3.3.4 Conclusion

The experiments using the bystander suppression assay suggest that the immunoregulatory cell responsible for oral tolerance in KLH-fed group 3 may act to suppressor the immune response to an unrelated antigen. Thus, the mechanisms by which it exerted its effect must be antigen independent. This experimental work needs to be confirmed.

7.3.4 FACS Scanning Data

7.3.4.1 Rationale and Aims of Experiments Using FACS Scanning

The results of feeding KLH to human volunteers altered the immune response to subsequent systemic immunisation to that antigen and led me to hypothesise that immunoregulatory cells were induced by feeding. Furthermore, the studies using the bystander suppression assay raised the possibility that a cell type with suppressor properties was induced. Animal studies have suggested several cell types that have such properties including CD4+ cells[115], CD8+ cells[110] and γδ cells[118]. I was interested in defining the physical characteristics of the immunoregulatory cell induced in my experiments as a step towards defining the nature of this cell.

I developed an experimental protocol to attempt to identify the cells that proliferate in lymphocyte culture using FACS scanning. As described in the Materials and Methods section, mononuclear cells were obtained from volunteers before exposure to KLH, after feeding and after immunisation. These cells were then cultured with 5μg/ml of KLH for 92 hours. These cells were then incubated with the appropriate monoclonal antibodies before being examined in the flow cytometer. I hypothesised that any KLH specific lymphocytes would be activated and proliferate in response to KLH in culture and that they could then be identified with labelled antibodies to proliferation markers. The proliferation markers that were chosen were CD25, CD38, CD45RO and HLA-DR. The morphology of these activated cells could then be characterized by co-staining with antibodies to cell surface markers labelled with a different chromogen, and I initially chose to investigate whether activated cells expressed CD4 or CD8.

7.3.4.2 Results

Four volunteers from the control group and four volunteers from KLH-fed group 3 were included in this experiment. An example of the results obtained is shown in figure 7.5. Figure 7.5a illustrates the initial scatter diagram of size (forward scatter) against granularity (side scatter). The lymphocytes can be identified by their position on the figure and are highlighted in red. The level of staining of PE and FITC on these selected cells can be plotted alone as shown in figure 7.5b. This example shows the level of CD25, stained with FITC-labelled monoclonal antibody, plotted against the level of CD4, stained with PE-labelled monoclonal antibody. Immunisation resulted in no detectable increase in the level of staining of any activation marker compared to baseline in either the control group or the pre-fed group after feeding or after immunisation. There was no difference in the frequency distribution of cells demonstrating differing amounts of activation markers before and after encounter with KLH in either the control or pre-fed group. Figure 7.5c illustrates an example comparing the frequency distribution of CD25 before and after immunisation of a volunteer in the control group and again no difference in frequency distribution could be detected.

Figure 7.5a – typical dot plot showing the morphological characteristics of isolated lymphocytes after 96 hours in culture. The gated cells in red represent lymphocytes. The smaller "cells" are likely to represent cellular debris.

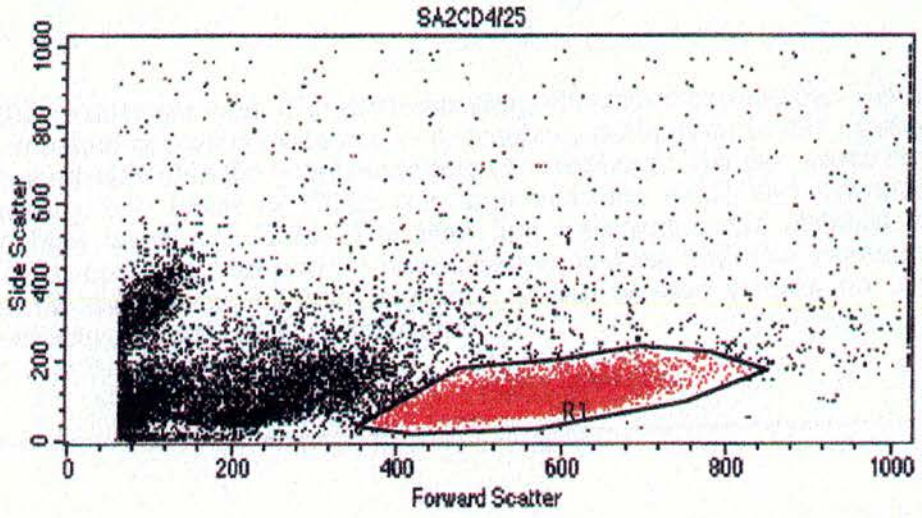


Figure 7.5b – dot plot of the gated lymphocytes showing the surface expression of CD4 vs. CD25. The cell population in the upper right hand quadrant represent a cell population with positive surface expression of both markers and therefore are activated CD4+ lymphocytes. Quadrant characteristics can be calculated and compared at different times within or between groups.

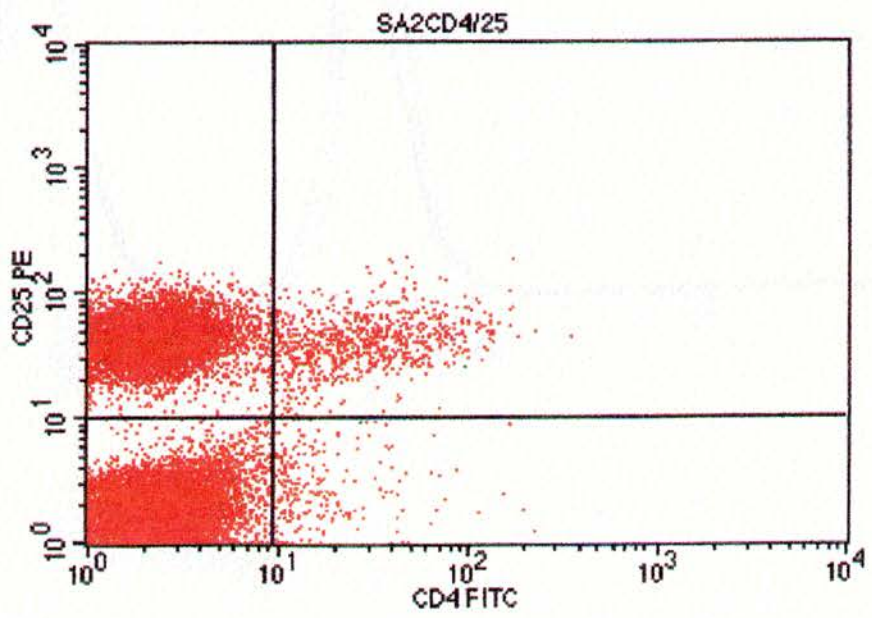
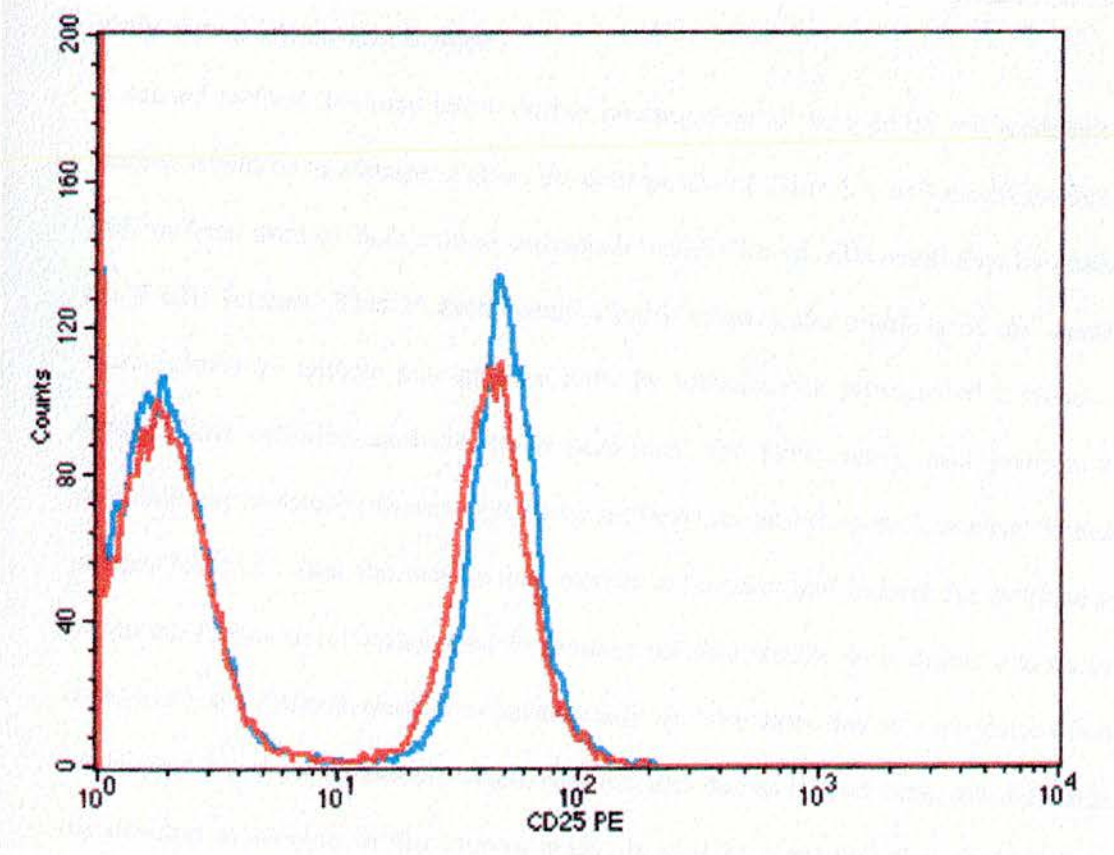


Figure 7.5c – a different method of demonstrating cell surface expression is shown. The amount of CD25 expressed per cell is expressed as a frequency histogram. In this example, the cells counted are all from within the gated area shown above. The two peaks represent cells expressing low levels of CD25 (i.e. non activated cells) and high levels of CD25. The blue line represents cells obtained from a volunteer in the control group prior to immunisation, and the pink line represents cells from the same volunteer after immunisation. As can be seen, there is no increased frequency of cells expressing high levels of CD25 after immunisation.



7.3.4.3 Discussion

This experiment was designed to identify cells that had been activated in the lymphocyte proliferation assay, and then define the morphological characteristics of these cells. The cell activation markers used to identify activated cells were antibodies to CD25, CD38, CD45RO and HLA-DR labelled with FITC. CD25 is the IL-2 receptor and is found on activated T cells, B cells and monocytes. In addition, CD25 is said to be down regulated on anergic cells and therefore, if this assay worked well, I may have been able to use this marker to assess for the presence of anergy. HLA-DR is also up-regulated on activated T cells and macrophages[191]. CD38 is found on activated T cells and on plasma cells[192]. CD45, otherwise known as leucocyte common antigen, is found on all leucocytes. It is present in two isoforms – CD45RA which is found on virgin T cells and CD45RO which is found on primed, activated T cells[193]. The experiments described were designed to assess whether the immunoregulatory cells were of CD4+ phenotype, which would be consistent the production of T_R1 or T_H3 cells by feeding, or of CD8+, which implies the production of a suppressor cell. PE-labelled anti-CD4 and anti-CD8 were therefore added to define the phenotype of any activated cells identified by the above markers.

The baseline data would have been used as a control group to ensure that the cells were specifically activated by KLH. Unfortunately, I was unable to detect any rise in activation markers over baseline and so the experiment failed to yield any positive results.

There are a number of possible reasons for my failure to detect activated cells in this assay. Firstly it is likely that the number of activated lymphocytes is so small in comparison to the total number of lymphocytes in culture that no statistical differences can be detected. An alternative explanation is that immunoregulatory cells may not express the activation markers that I assayed for, although the wide panel of activation markers that I used makes this less likely. Lastly lymphocytes activated in this culture system may have an abnormal size and granularity and therefore be out-with the gated area chosen initially. To allow for

this possibility, the data was re-analysed without using the initial gating, and again no differences were seen.

Two possible methods may increase the sensitivity of this technique and may be worthy of further study. Firstly, it may be possible to stain the nuclei with propidium iodide. This stain can allow the FACS scanner to identify the stage of the cell cycle that each cell is in. The computer software can therefore be instructed to run its analysis on actively dividing cells only. Antigen specific lymphocytes that are stimulated by antigen should be dividing and therefore running the analysis on these cells may improve the sensitivity of the test. I made an attempt to develop this technique, but did not successfully optimise the assay in time to use it in these experiments.

A second technique that may help identify the immunoregulatory cell would be to clone the cell. Using a population of cloned cells in the FACS scanner would also reduce the number of lymphocytes that are not reactive to KLH and therefore increase the sensitivity of this technique. Furthermore, it may be easier to identify the cytokine profile of the immunoregulatory cell from a cloned population. Cloning is a well established technique and has been used on both animal and human cells. There are however problems with cloning. The cloning process itself can alter the expression of cell surface marker and indeed the cytokine profile produced which may give unreliable results. In addition, it is a technically demanding and difficult method to develop and my laboratory has no experience of cloning techniques. For a combination of these reasons, and due to lack of time, I did not attempt to develop clones of these cells. It may be a method that could be usefully incorporated into future studies to identify the cells responsible for tolerance production in humans.

7.4 Evidence for Mechanisms of Tolerance in OVA Group

7.4.1 Summary of Results of Experiments Using OVA

Immunisation with OVA resulted in no detectable DTH response or *in vitro* lymphocyte proliferation. There was a low level of anti-OVA IgG and IgA in the serum of volunteers before immunisation, but after lifelong exposure to OVA in the diet. Low dose immunisation did not provoke a rise in levels of either of these antibodies. The four volunteers who received the higher dose immunisation schedule showed no, or a very modest, increase in antibody levels. I have argued that these results would be consistent with the induction of clonal anergy or clonal deletion to prolonged exposure to orally encountered antigen

7.4.2 Absence of Bystander Suppression Induced by OVA

The bystander suppression assay has been described earlier in this chapter. It is possible that the powerful OVA specific suppressor cells may have been induced by feeding and inhibit the production of humoral or CMI, particularly if these cells are anergic i.e. do not proliferation in response to OVA. I therefore used the previously described assay to assess whether the addition of OVA to cells obtained from OVA immunised volunteers inhibited the response to PPD. A suppression index was calculated as previously described.

Samples from 10 volunteers were run on this assay. Samples from before the immunisation schedule (i.e. after oral exposure to OVA) and after immunisation were included. There was no evidence of bystander suppression in any of the samples (data not shown). These results do not support the hypothesis that active suppression is an important mechanism in maintaining oral tolerance to OVA.

7.4.3 Reversal of Anergy with IL-2

7.4.3.1 Introduction and Rationale

Clonal anergy is a powerful mechanism of tolerance production and can be defined as a long-lived state of reduced responsiveness to antigen by antigen specific lymphocytes[86]. Clonal anergy has been shown to be responsible for oral tolerance in animal models under certain feeding conditions[144;145]. The complete absence of CMI responses and the very sluggish antibody response to OVA immunisation would be consistent with the induction of clonal anergy by prolonged OVA ingestion.

One experimental method that has been used to imply the presence of anergy in animal models of oral tolerance has been to demonstrate the reversal of tolerance by culturing anergic lymphocytes with IL-2[148]. I attempted to demonstrate reversal of anergy in *in vitro* lymphocyte proliferation assay by preculturing lymphocytes with IL-2.

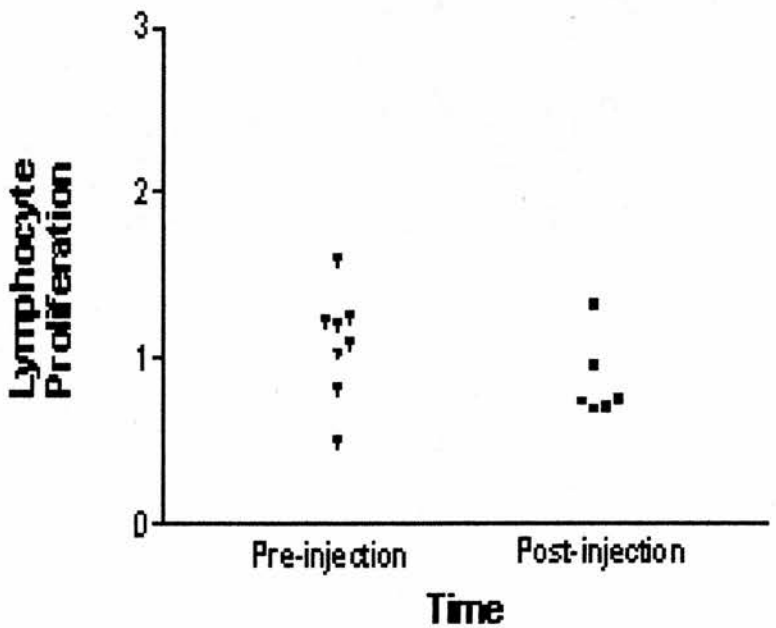
7.4.3.2 Results

The results are shown in figure 7.6. As can be seen, there was no evidence of lymphocyte proliferation to OVA following pre-culture with IL-2 in cells obtained before or after the injection schedule. Interestingly, addition of this dose of IL-2 to cells cultured for 96 hours without antigen stimulation induces an impressive 15-fold increase in lymphocyte proliferation compared to cells cultured without IL-2 (data not shown).

7.4.3.3 Discussion

These experiments did not provide any evidence to support the hypothesis that clonal anergy is responsible for tolerance to OVA in humans. There are two possible explanations. Firstly, clonal anergy may not be the cause of tolerance and another mechanism such as clonal deletion may be responsible. Alternatively, clonal anergy may be the underlying mechanism of tolerance, but is not reversed by IL-2 in this assay.

Figure 7.6 – Results of lymphocyte proliferation to OVA after pre-culture with IL-2. There was no evidence of proliferation from cells taken from volunteers either before of after immunisation.



There are a number of other technical reasons that may explain why my assay failed. Firstly, the IL-2 may not be biologically active in the dose I added. This is unlikely since I used the same dose as reported in previous, successful animal studies[144]. The observation that the addition of IL-2 cells cultured without antigen stimulation induces a 15-fold increase in lymphocyte proliferation compared to cell cultured without IL-2 suggests that IL-2 is biologically active in the concentration used.

Other potential reasons for failure to reverse tolerance may be that the APCs adhered to the wells at the first stage of culture and were therefore not transferred with the lymphocytes to the second stage of culture, or that they did not survive culture with IL-2. If no APCs were present in the second stage of culture, lymphocytes would not proliferate even if anergy had been reversed. Three strategies were used to ensure APCs were present in the second stage of culture. Firstly, in eight subjects a cell scraper was used to ensure all possible cells were transferred from the first to the second stage of culture. Secondly, lymphocytes were cultured in the wells to be used for the second stage of culture from the start of the experiment on four occasions. After the first stage of the experiment, the supernatants from these wells were removed. If adherence of APCs is the reason for failure of this assay, then functional APCs should have remained in the wells for the second stage of the experiment. Lastly, to test for the possibility that APCs did not survive the two-stage culture process, fresh mononuclear cells were collected from two volunteers between the two stages of the experiment and used in the second stage of culture. None of these modifications to the experimental technique resulted in any detectable lymphocyte proliferation to OVA.

Another potential explanation for the failure to demonstrate reversal of anergy may be due to differences in the protocol between previously reported experiments on animal models and my experiments on humans. Firstly, the feeding regime used in the animal experiments gave 1 large dose of antigen[144;148]. Conversely, the volunteers in my study received a lower dose per weight over a much more prolonged period. It may be that human OVA specific lymphocytes are more profoundly anergic and therefore do not respond to IL-2. A second

important difference is that the animal studies obtained cells from the spleen[144], whereas I used peripheral blood cells. Anergic cells may reside in the spleen and not be present in the peripheral circulation, even after immunisation. Therefore, the difficulties of obtaining lymphocytes from tissue other than blood in humans may be the explanation for my failure to demonstrate reversal of anergy with IL-2.

Lastly, the way I chose to demonstrate the reversal of anergy in my experiments differs from that used in animal studies. Animal studies used adoptive transfer of IL-2 pre-cultured cells into irradiated mice to demonstrate the return of function. They showed that these mice were able to produce antibody. This may be an important difference since anergic cells can secrete cytokines and may therefore be able to provide help to antibody production, without being able to proliferate[190]. Thus my method of showing reversal of anergy may not be as sensitive as these methods. Again, it is impossible to reproduce these animal experiments in humans.

In summary, these experiments did not provide supportive evidence for the presence of clonal anergy to OVA in humans. However, this may be due to problems obtaining appropriate samples from humans or in assay techniques that mean that the initial hypothesis cannot be rejected on this evidence and further work in this area is required.

8 GENERAL DISCUSSION

8.1 Discussion of Results Obtained from KLH Experiments

8.1.1 Overview of Results of the Induction of Tolerance with KLH

KLH, chosen because of its availability and proven safety in humans, was used to investigate low dose tolerance in humans. An actively immunised control group demonstrated a good cell mediated immune response, including positive DTH responses and lymphocyte proliferation to KLH *in vitro*, and humoral responses. Three different feeding regimes of KLH were given to groups of volunteers, before their immune system was challenged by systemic immunisation with KLH. Any differences between the immune response in the control group and the pre-fed groups were attributed to oral tolerance.

KLH-fed group 1 ingested 10mg of KLH daily for 10 consecutive days. There was no difference between the immune response in this group and the control group, which suggests that a minimum dose of feeding is required to induce detectable changes in the systemic immune responses.

The feeding regime given to KLH-fed group 2 was 50mg of KLH daily for 10 consecutive days. When compared to the control group, there was a reduced DTH response associated with an unchanged lymphocyte proliferation response after immunisation. The relationship between DTH and *in vitro* lymphocyte proliferation that was seen in the control group was lost. Furthermore IgG, but not IgA, antibody responses to KLH were primed. Feeding alone induced a detectable *in vitro* lymphocyte proliferation response, but did not induce a detectable humoral response.

Another group, KLH-fed group 3, was given a more prolonged course of feeding of 50mg of KLH for 10 consecutive days, a gap of five days followed by a further five daily 50mg feeds. Following this feeding regime there was a detectable *in vitro* lymphocyte proliferation response, but no detectable humoral response. Again, the relationship between DTH and *in*

vitro lymphocyte proliferation that was seen in the control group was lost. Following immunisation, the immune response was similar to that seen in KLH-fed group 2 in that there was a reduced DTH response and a detectable *in vitro* lymphocyte proliferation response. IgG responses were not significantly raised compared to baseline. The immune responses between KLH-fed group 2 and KLH-fed group 3 was different in two respects. Firstly, the IgG response after immunisation was no longer raised in KLH-fed group 3 compared to the control group. Secondly the *in vitro* lymphocyte proliferation response was significant higher after feeding but significantly reduced after immunisation in KLH fed-group 3 compared to group 2.

8.1.2 Hypotheses Drawn from Experiments on Tolerance Induction with KLH

The results for KLH-fed group 1 indicate that there is a minimum level of feeding that is necessary to induce detectable changes in the systemic immune response. There may have been a low level immune response that was not detectable by the assays that I used and it would be interesting to rechallenge these volunteers to see if any subsequent immune response would be brisker or more complete compared to totally naïve controls.

The results from KLH-fed groups 2 and 3 convincingly demonstrate that tolerance to DTH responses have been induced by pre-feeding KLH, but than tolerance in the humoral compartment of the immune system has not occurred. Priming of the IgG response associated with suppression of DTH to the fed antigen has been observed in animal studies[14;16;194]. A suppressor cells was identified by transfer experiments as the mechanism of tolerance[16]. The results obtained from the experiments with KLH-fed group 2 are similar to these studies and I therefore hypothesised that an immunoregulatory cell is responsible for tolerance in my studies. I suggested that proliferation of this cell, induced by feeding, is responsible for the detectable *in vitro* lymphocyte proliferation after feeding. This cell subsequently modifies the immune response to KLH immunisation to suppress the DTH

responses but enhance anti-KLH IgG production. Putative immunoregulatory cells that may have these properties include T_H2 cells[58] and T_R1 cells[139].

The differences in the immune response in KLH-fed group 3 show more pronounced tolerance than KLH-fed group 2 in that the post immunisation *in vitro* lymphocyte proliferation responses are reduced. In addition both anti-KLH IgG and IgA levels are reduced after immunisation in the KLH-fed group 3. This pattern has also been seen in animal studies in which a suppressor or immunoregulatory cell was identified[99] I hypothesised that such a cell was also responsible for feeding in KLH-fed group 3. Feeding alone causes an increase in *in vitro* lymphocyte proliferation compared to KLH-fed group 2, but there is no further priming of the IgG response. I hypothesised that these results were not consistent with an increased T_H2 response but may be consistent with the induction of T_R1 cell[139] or T_H3 cells[133]. Although I chose to investigate the hypotheos that immunoregulatory cells were responsible for tolerance to KLH, an alternative hypothesis that would be consistent with the data is that clonal anergy[141] or clonal deletion[133] may have been induced by feeding.

8.1.3 Experiments Designed to Investigate these Hypotheses

Chapter 7 details the results of several lines of investigation that I performed to investigate these hypotheses. The results for these experiments failed to provide definitive evidence for the presence of these putative immunoregulatory cells. This may be because the experiments designed were not adequate to detect them, or because the hypotheses are wrong and that different mechanisms are in fact responsible for tolerance induction to KLH. In this section, I will discuss the limitations of each of the experiments performed.

8.1.3.1 Criticisms of Investigations of Cytokine Production

8.1.3.1.1 ELISA Methods of Cytokine Measurement

Commercially available kits, complete with positive controls were purchased. The positive controls gave positive results indicating the kits had worked. However, only three specimens gave any detectable results for IFN-γ and all were at or below the lowest value of the positive control. No IL-4 was detected in any test sample. The reason for the failure of this experiment is likely to be because the amount of cytokine in the supernatent of each sample is below the detection limit for the assay. Husby *et al* reported similar problems in their experiments on oral tolerance in humans[9]. If further work is performed using such assays, samples stimulated with conA, which causes a good proliferation response, should be included as an additional positive control.

8.1.3.1.2 PCR Methods to Detect Cytokine mRNA

The failure of the ELISA methods to detect cytokines led me to develop the PCR based assays. These have the advantage of being very sensitive. There are several drawbacks, including the fact that they measure a surrogate marker of cytokine levels (i.e. mRNA) and that they do not give quantatitive results. I was able to detect cytokine mRNA but the results were inconclusive.

The addition of further controls may have be helpful to improve our ability to interpret these results. Firstly samples obtained from cells cultured without KLH at all stages of the assay should have been included. This would have allowed direct comparisons between positive and negative samples cultured at the same time to have been made. Secondly further positive controls with samples of cells known to produce specific cytokine mRNA at high levels would have been helpful. The positive controls that I used were stimulated with conA and the levels of each cytokine mRNA that these controls produce is unknown.

PCR methods have shown some promise in my hands as I was able to detect cytokine mRNA. Further work incorporating the above controls would be helpful. Such work should also attempt to use quantitative PCR techniques to allow direct comparison between groups.

8.1.3.2 Criticisms of FACS Scanning Experiments

The FACS scan experiments were designed to identify the immunophenotypic characteristics of the cells that gave positive results in the *in vitro* lymphocyte proliferation assays. I had hypothesised that the cells that proliferated in the lymphocyte proliferation assay may be immunoregulatory cells and I was interested in characterising them.

The assay failed to detect the proliferating cells and therefore I could not characterise them. There were a number of potential problems with this assay. Firstly, the population of antigen specific cells are a very small proportion of the total number of lymphocytes within the circulation. Therefore it may be very difficult for techniques such as these to detect any population of antigen specific lymphocytes. Furthermore it is not known whether the surface markers used to detect dividing cells (i.e. CD25, CD38, CR45RO and HLA-DR) are upregulated on immunoregulatory cells in humans and therefore whether the FACS scan could detect these cells. Finally, there were no positive controls built into the experimental design. Therefore it is impossible to know whether the negative results obtained in this experiment are due to the experimental technique or due to the fact that there were in fact no immunoregulatory cells present. In addition, the negative controls used were cells obtained from volunteers prior to encounter with KLH. The addition of cells taken at each time point during the experiment and cultured without KLH would have been a useful additional negative control.

For the above reasons, it may be very difficult to get positive results with experimental techniques such as these. If they are used in the future, attempts to include positive controls should be made. Cells cultured with conA to provoke more generalised T-cell proliferation would perhaps be one such control. Other methods to improve the sensitivity of the

technique should be identified. One such possibility would be to stain the cells with propidium iodide, which identifies actively dividing cells and then limit the analysis to these cells.

8.1.3.3 Criticisms of the Bystander Suppression Assay

These results implied that there may be regulation of a different immune response by cells induced by feeding KLH, which would suggest the presence of an immunoregulatory cell. These results were obtained on a very small sample size and therefore should be interpreted with caution, but further work to reproduce these results is warranted. The experiments are limited by the lack of a positive control (i.e. known immunoregulatory cells that induce bystander suppression) to ensure that the assay can indeed detect bystander suppression. Finding such controls would be very difficult, although valuable. In addition in my experiments I used an already established immune response (i.e. PPD). This was intentional because I did not want to introduce the potential confounder of giving two immunisation schedules simultaneously. However the use of an immune response established by immunisation earlier in life leads to potential problems in that the degree of active immunity of each individual tested may vary. Further experimental protocols using two separate neo-antigens, one to induce oral tolerance and one to assess bystander suppression, would be a useful way to extend this work.

8.1.4 Conclusions from Experiments on Tolerance to KLH

The results from the KLH experiments confirm the earlier observations that oral tolerance to a neo-antigen can be induced to DTH by short course feeding.

I hypothesised that tolerance may be due to the production of an immunoregulatory cell. I failed to find any direct evidence of such a cell. As the above discussion emphasises, the experimental methods used may not have been adequate to detect such cells and the absence of evidence cannot be taken for evidence of absence. However, it is possible that the initial

hypothesis was wrong, and that mechanism of tolerance induction other than immunoregulatory cells are induced. One possibility is that the absence of DTH responses are the result of T-cell anergy and that feeding primes B-cell responses. Recent work suggesting that anergic cells can have suppressor functions[155;156] would be consistent with this hypothesis. Further work on oral tolerance in humans should incorporate protocols to investigate the presence of such mechanisms of tolerance.

8.2 Discussion of Results Obtained from OVA Experiments

8.2.1 Overview of Results of the Investigation of Tolerance to OVA

Animal studies suggest that high dose feeding results in the induction of different mechanisms of tolerance such as clonal anergy[141;143] and clonal deletion[133]. To assess whether similar mechanisms may occur in humans, I used a similar protocol to assess the systemic immune response to OVA. OVA was chosen because it is a common dietary antigen, a source of OVA that was safe for injection was available and our laboratory has experience of assaying for anti-OVA antibodies. The results gave an entirely different pattern of immune responses compared to the KLH-fed groups. There were no detectable cell-mediated immune responses at any time. Low levels of anti-OVA IgG and IgA were detected at baseline, but there was a very poor humoral response to systemic immunisation.

8.2.2 Hypotheses Drawn from Experiments on Tolerance to OVA

Tolerance to OVA affects both humoral and cell mediated immunity. This more complete tolerance lead me to hypothesise that more powerful, additional mechanisms of tolerance may be involved in maintaining tolerance to OVA. One such mechanism would be clonal anergy. I attempted to develop an assay using IL-2 to reverse tolerance to test this hypothesis.

8.2.3 Criticisms of Experiments Using OVA

The major criticism of the experiments using OVA is the lack of a positive control. All test subjects have encountered OVA regularly in their diet and therefore no group is available to assess the immune response to OVA in naïve controls. In is thus impossible to know whether the lack of immune response to OVA is because OVA itself is not immunogenic or because oral tolerance has been induced. This has been discussed more fully in chapter 6. I attempted to circumvent this problem by increasing the immunisation dose used. Further work on oral tolerance to prolonged feeding with antigen should address this problem. Two possible methods are available. Firstly a dietary antigen such as OVA could be used, but the immune system should be challenge by immunisation containing adjuvant. This would increase the immunogenicity of the injections, but there would still be no control group. An alternative method for which a control group could be obtained would be to give prolonged feeding with a neo-antigen such as KLH. This kind of protocol has the drawbacks of being expensive and being difficult for the volunteer subjects to follow.

OVA was used to investigate high dose tolerance, and it is true that the volunteers would have consumed large quantities of OVA over their lifetimes. However, the amount of OVA ingested is uncontrolled and the total dose consumed by each volunteer may vary wildly. Furthermore, in the animal models of high dose tolerance that identified the mechanisms of clonal anergy and clonal deletion, a single large dose feed was given[133;141]. Therefore the use of a dietary antigen to investigate this type of high dose tolerance may be inappropriate. Further studies using single, or short course, feeds of neoantigen such as KLH in larger doses than used in my experiments would be required to investigate this type of tolerance.

I failed to find direct evidence for anergy by IL-2 reversal. There are several differences between the protocol I developed and those used in animal studies that may explain this failure. These include the fact that different feeding schedules were given and that the cells cultured with IL-2 in animal studies were obtained from the spleen and not the peripheral circulation[144]. Therefore it is impossible to know whether failure of the assays or a true

absence of clonal anergy is responsible for the negative results obtained. These difficulties are compounded by the lack of a positive control sample – i.e. known anergic cells in which a return of function can be demonstrated in this assay.

8.2.4 Conclusions from Experiments on Tolerance to OVA

There was a marked lack of immune response to OVA after both the low and high dose immunisation schedules. The experiments that I performed cannot differentiate conclusively whether this is due to the presence of powerful mechanisms of tolerance induction or to the fact that the OVA immunisation schedule was not immunogenic. I did hypothesise that clonal anergy may be responsible for the maintenance of tolerance but no evidence could be obtained to support this hypothesis.

8.3 Difficulties Encountered During these Experiments on Humans

In this thesis, the KLH experiments successfully demonstrated that oral feeding alters the systemic immune response to that antigen although I was unable to define the nature of the tolerance. There are a number of difficulties in developing experimental techniques to investigate mechanisms of tolerance in human studies that do not apply to animal work that make identifying such mechanisms problematic. Problems encountered include recruiting large numbers of well-motivated volunteers to take part in these trials. Furthermore, human study groups are heterogeneous leading to a potential wide variability in their immune responses. Therefore, in many experiments, one has small groups with a wide range of values thus reducing the chance of obtaining a statistically significant result. Experiments on animal strains overcome both these difficulties. There are also limitations on the type of experiments that can be designed. For example, it is almost impossible to biopsy interesting tissue such as mesenteric lymph nodes or spleen. For ethical reasons, the immune response of the volunteers cannot be manipulated *in vivo*, by, for example, giving monoclonal antibody to specific cytokines to investigate their effect on the immune process. Similarly

transfer experiments, which have been used to identify suppressor cells in animal models[13;32;100], are impossible in humans. The investigation oral tolerance in humans is therefore a real scientific challenge.

8.4 Ideas for Further Research

Further work on oral tolerance in humans needs to be extended in two ways. Firstly the range of tolerance needs to be delineated. The effect of various feeding schedules, in terms of length of feeding and dose of feeds, on the immune response have to be defined. In addition tolerance to different types of antigen, such as other soluble proteins, particulate antigens and even bacteria, have to be investigated, as has occurred in animal models[14;28]. Finally, research on the length of time that tolerance persists after feeding is required.

Secondly further work on the nature of the mechanisms of tolerance in humans is required. The sections above illustrate many of the difficulties that will be encountered in such research. *In vitro* assays, such as the bystander suppression assay, need to be developed and validated. Manipulations of such assays, for example by adding specific cytokines or anti-cytokine monoclonal antibodies, will be required if the molecular basis for oral tolerance in humans is to be elucidated.

8.5 Epilogue

In this thesis I have developed a method to demonstrate the presence of oral tolerance in humans. I have used this method to investigate the effects of feeding different doses of soluble protein antigen. I have shown that oral tolerance by low dose feeding can be induced and inhibits DTH response. Investigations of the immune response to a common dietary antigen suggests that tolerance is more complete to these antigens, although further studies will be required to confirm these observations. The knowledge of oral tolerance in humans is analogous to early animal work when investigators were defining the extent of oral tolerance in animals. Further work to investigate the extent and nature of oral tolerance in humans is

required before clinical applications of oral tolerance in the treatment of autoimmune diseases and the investigation of the aetiology of gastrointestinal inflammation can be extended to humans.

Reference List

1. Dakin R: Remarks on a cutaneous affection produced by certain poisonous vegetables. American Journal of Medical Sciences 4:98-100, 1829

2. Wells HG, Osbourne TB: The biological reactions of the vegetable proteins. Journal of Infectious Diseases 8:66-124, 1911

3. Wells HG: Studies on the chemistry of anaphylaxis. III. Experiments with isolated proteins, especially those of the hen's egg. Journal of Infectious Diseases 9:147-171, 1911

4. Chase MW: Inhibition of Experimental Drug Allergy by prior feeding of the Sensitizing Agent. Proceedings of the Society for Experimental Biology & Medicine 61:257-259, 1946

5. Glaister JR: Some effects of oral administration of oxazolone to mice. International Archives of Allergy 828-843, 1973

6. Challacombe CM, Tomasi TB: Systemic tolerance and secretory immunity after oral immunization. J.Exp.Med. 152:1459-1472, 1980

7. Mowat AM: Oral tolerance and regulation of immunity to dietary antigens, in Handbook of Mucosal Immunology. (ed): 16. Academic Press, Inc., 1994, pp 185-201

8. Stokes CR, Miller BG, Bourne FJ: Animal models of food sensitivity, in Brostoff J, Challacombe SJ. (eds): Animal models of food insensitivity. Eastbourne, Saunders, 1987, pp 286-300

9. Husby S, Mestecky J, Moldoveanu Z, et al: Oral Tolerance in Humans. T cell but not B cell tolerance after antigen feeding. Journal of Immunology 4663-4670, 1994

10. Heppell LMJ, Kilshaw PJ: Immune responses of guinea pigs to dietary protein I Induction of tolerance by feeding with ovalbumin. Int.Arch.Allergy Appl.Immunol. 68:54-59, 1982

11. MacDonald TT: Immunosuppreesion caused by antigen feeding II. Suppressor T- cells mask Peyer's patch B cell priming to orally administered antigen. Eur.J.Immunol. 13:138-142, 1983

12. O'Mahony S, Arranz E, Barton JR, et al: Dissociation between systemic and mucosal humoral immune responses in coeliac disease. Gut 32:29-35, 1991

13. Asherson GL, Zembala M, Perera MACC, et al: Production of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissues. Cellular Immunology 33:145-155, 1977

14. Kagnoff MF: Effects of antigen feeding on intestinal and systemic immune responses III. Antigen-specific serum mediated suppression of humoral antibody responses after antigen feeding. Cellular Immunology 40:186-203, 1978

15. Saklayen MG, Pesce AJ, Pollak VE, et al: Kinetics of oral tolerance: study of variables affecting tolerance induced by oral administration of antigen. International Archives of Allergy & Applied Immunology 73:5-9, 1984

16. Peng HJ, Turner MW, Strobel S: The kinetics of oral hyposensitization to a protein antigen are determined by immune status, dose and frequency of antigen administration. Immunology 67:425-430, 1989

17. Strobel S, Ferguson A: Persistence of oral tolerance in mice fed ovalbumin is different for humoral and cell-mediated immune responses. Immunology 317-318, 1987

18. Peri BA, Rothberg RM: Circulating antitoxin in rabbits after the ingestion of diphtheria toxin. Infection and Immunity 32:1148-1154, 1981

19. Korenblat PE, Rothberg RM, Minden P, et al: Immune responses of human adults after oral and parental exposure to bovine serum albumin. Journal of Allergy 41:226-235, 1968

20. Richman LK, Graeff AS, Yarchoan R, et al: Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. Journal of Immunology 126:2079-2083, 1981

21. Wilson AD, Stokes CR, Bourne FJ: Adjuvant effect of cholera toxin on the mucosal immune response to soluble protein. Differences between mouse strains and protein antigens. Scand.J.Immunol. 29:739-745, 1989

22. Elson CO, Ealding W: Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. Journal of Immunology 133:2892-2897, 1984

23. Holmgren J: Actions of cholera toxic and the prevention and treatment of cholera. Nature 292:413-417, 1981

24. Van der Heijden PJ, Bianchi ATJ, Dol M, et al: Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice. Immunology 72:89-93, 1991

25. Ploix C, Bergerot I, Durand A, et al: Oral administration of cholera toxin B-insulin conjugates protects NOD mice from autoimmune diabetes by induceing CD4+ regulatory cells. Diabetes 48:2150-2156, 1999

26. Sun J-B, Holmgren J, Czerkinsky C: Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. Proc.Natl Acad.Sci.USA 91:10795-10799, 1994

27. Duchmann R, Kaiser I, Mayet W, et al: Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clinical & Experimental Immunology 102:448-455, 1995

28. Karlsson MR, Kahu H, Hanson LA, et al: Neonatal colonization of rats induces immunological tolerance to bacteria antigens. Eur.J.Immunol. 29:109-118, 1999

29. Santos LMB, Al-Sabbagh A, Londono A, et al: Oral tolerance to myelin basic protein induces regulatory TGF-β secreting T cells in Peyer's patches of SJL mice. Cellular Immunology 157:439-447, 1994

30. Zhang ZJ, Lee CSY, Lider O, et al: Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. Journal of Immunology 145:2489-2493, 1990

31. Lamont AG, Mowat AM, Parrott DMV: Priming of systemic and local delayed-type hypersensitivity responses by feeding low doses of ovalbumin to mice. Immunology 66:595-599, 1989

32. Miller SD, Hanson DG: Inhibition of specific immune responses by feeding protein antigens IV. Evidence for tolerance and specific active suppression of cell-mediated immune response to ovalbumin. Journal of Immunology 123:2344-2350, 1979

33. Kagnoff MF: Effects of antigen-feeding on intestinal and systemic immune responses II. Suppression of delayed-type hypersensitivity reactions. Journal of Immunology 120:1509-1513, 1978

34. Ngan J, Kind LS: Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. Journal of Immunology 120:861-865, 1978

35. Panja A, Blumberg RS, Balk SP, et al: CD1d is involved in T cell-intestinal cell interactions. J.Exp.Med. 178:1115-1119, 1993

36. Strobel S, Ferguson A: Immune responses to fed protein antigens in mice. 3. Systemic tolerance or priming is related to the age at which an antigen is first encountered. Ped.Res. 18:588-593, 1984

37. Lamont AG, Bruce MG, Watret KC, et al: Suppression of an established DTH response to ovalbumin in mice by feeding after immunisation. Immunology 64:135-139, 1988

38. Lafont S, Andre C, Andre F, et al: Abrogation by subsequent feeding of antibody responses, including IgE, in parenterally immunized mice. J.Exp.Med. 155:1573-1578, 1982

39. Chung Y, Chang S-Y, Kang C-Y: Kinetic analysis of oral tolerance: memory lymphocytes are refractory to oral tolerance. Journal of Immunology 163:3692-3698, 1999

40. Melamed D, Friedman A: Modification of the immune response by oral administration: antigen requirements and interaction with immunogenic stimulus. 146 4201993

41. Stokes CR: Induction and control of intestinal immune responses, in Stokes CR, Newby TJ. (eds): Local immune responses of the gut. Boca Raton, Florida, C.R.C. Press, 1984, pp 97-141

42. Peng HJ, Turner MW, Strobel S: Failure to induce oral tolerance to protein antigens in neonatal mice can be corrected by transfer of adult spleen cells. Ped.Res. 26:486-490, 1989

43. Strobel S: Dietary manipulation and induction of tolerance. Journal of Pediatrics 121:S75-S791992

44. Sanderson IR, Ouelette AJ, Carter EA, et al: Ontogeny of Ia messenger RNA in the mouse small intestine epithelium is modulated by age of weaning and diet. Gastroenterology 105:974-980, 1993

45. Wannemuehler MJ, Kiyono H, Babb JL, et al: Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germ-free mice to sensitivity to oral tolerance. Journal of Immunology 129:959-965, 1982

46. Moreau M-C, Corthier G: Effect of the gastrointestinal microflora on induction and maintenence of oral tolerance to ovalbumin in C3H/HeJ mice. Infection and Immunity 56:2766-2768, 1988

47. Khoury SJ, Lider O, Al-Sabbagh A, et al: Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein III. Synergistic effect of lipopolysaccharide. Cellular Immunology 131:302-310, 1990

48. Jenkins MK: Models of lymphocyte activation. Immunology Today 13:69-73, 1992

49. Bretscher P, Cohn M: A Theory of Self-Nonself Discrimination. Science 169:1042-1049, 1970

50. Mizel SB, Ben-Zvi A: Studies on the role of lymphocyte-activating factor (interleukin 1) in antigen induced lymph node proliferation. Cellular Immunology 54:382-389, 1980

51. Weaver CT, Unanue ER: The costimulatory function of antigen-presenting cells. Immunology Today 11:49-55, 1990

52. Jenkins MK, Ashwell JD, Schwartz RH: Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen presenting cells. Journal of Immunology 140:3224-3330, 1988

53. Freeman GJ, Gray GS, Gimmi CD, et al: Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. J.Exp.Med. 174:625-631, 1991

54. Freeman GJ, Gribben JG, Boussiotis VA, et al: Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 909-911, 1993

55. Mueller DL, Jenkins MK, Schwartz RH: Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Ann.Rev.Immunol. 7:445-480, 1989

56. Yokochi T, Holly RD, Clark EA: B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus activated C cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. Journal of Immunology 128:823-827, 1992

57. Cherwinski HM, Schumacher JD, Brown KD, et al: Two types of mouse helper T cell clone III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridisation, functionally monospecific bioassays and monoclonal antibodies. J.Exp.Med. 166:1229-1244, 1987

58. Cher DJ, Mosmann TR: Two types of murine helper T cell clone II. Delayed-type hypersensitivity is mediated by Th1 clones. Journal of Immunology 138:3688-3694, 1987

59. Boom WH, Liano D, Abbas AK: Heterogenicity of helper/inducer T lymphocytes II. Effects of interleukin 4 and interleukin 2 producing T cell clones on resting B lymphocytes. J.Exp.Med. 167:1350-1363, 1988

60. Snapper CM, Paul WE: Interferon-g and B-cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236:944-947, 1987

61. Pfeifer JD, McKenzie DT, Swain SL, et al: B cell stimulatory factor (interleukin 4) is sufficient for the proliferation and differentiation of lectin-stimulated cytotoxic T lymphocyte precursors. J.Exp.Med. 166:1464-1470, 1987

62. Powrie F, Menon S, Coffman RL: Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. Eur.J.Immunol. 23:2223-2229, 1993

63. Rothenberg ME: Eosinophilia. New England Journal of Medicine 338:1592-1600, 1998

64. Street NE, Schumacher JH, Fong AT, et al: Heterogeneity of mouse helper T cells - evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. Journal of Immunology 144:1629-1639, 1990

65. Firestein GS, Roeder WD, Laxer JA, et al: A new murine CD4+ T cell subset with an unrestricted cytokine profile. Journal of Immunology 143:518-525, 1989

66. Rocken M, Saurat J-H, Hauser C: A common precursor for CD4+ T cells producing IL-2 or IL-4. Journal of Immunology 148:1031-1036, 1992

67. Gollob KJ, Dutra WO, Coffman RL: Early message expression of interleukin-4 and interferon-g, but not of interleukin-2 and interleukin-10, reflects later polarization of primary CD4+ T cell cultures. Eur.J.Immunol. 1565-1570, 1996

68. Nakamura T, Kamogawa Y, Bottomly K, et al: Polarisation of IL-4 and IFN-g producing CD4+ T cells following activation of naive CD4+ T cells. Journal of Immunology 158:1085-1094, 1997

69. Sedar RA, Paul WE, Davis MM, et al: The presence of interleukin 4 *in vitro* priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J.Exp.Med. 176:1091-1098, 1992

70. Swain SL, Weinberg AD, English M, et al: IL-4 directs the development of Th2-like helper effectors. Journal of Immunology 145:3796-3806, 1990

71. Betz M, Fox BS: Regulation and development of cytochrome c-specific IL-4 producing T-cells. Journal of Immunology 145:1046-1052, 1990

72. Fiorentino DF, Zlotnik A, Vieira P, et al: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. Journal of Immunology 146:3444-3451, 1991

73. Gajewski TF, Fitch FW: Anti-proliferative effect of IFN-γ in immune regulation. I IFN-g inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. Journal of Immunology 140:4245-4252, 1988

74. Fernandez-Botran R, Sanders VM, Mosmann TR, et al: Lymphokine-mediated regulation of the proliferative responses of clones of T helper 1 and T helper 2 cells. J.Exp.Med. 168:543-558, 1988

75. Constant SL, Bottomly K: Induction of TH1 and TH2 CD4+ T cell responses: The alternative approaches. Ann.Rev.Immunol. 15:297-322, 1997

76. Romagnani S: The Th1/Th2 paradigm. Immunology Today 18:263-266, 1997

77. Daugelat S, Kaufmann SHE: Role of Th1 and Th2 cells in bacterial infections. Chemical Immunology 63:66-85, 1996

78. Del Prete GF, De Carli M, Mastromauro C, et al: Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. Journal of Clinical Investigation 88:346-350, 1991

79. Maggi E, Del Prete G, Macchia D, et al: Profiles of lymphocyte activities and helper function for IgE In human T cell clones. Eur.J.Immunol. 18:1045-1050, 1988

80. Mosmann TR, Coffman RL: Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. Ann.Rev.Immunol. 7:145-173, 1989

81. Else KJ, Finkleman FD, Maliszewski CR, et al: Cytokine-mediated regulation of chronic intestinal helminth infection. J.Exp.Med. 179:347-351, 1994

82. Richman LK, Chiller JM, Brown WR, et al: Enterically induced immunological tolerance I. Induction of suppressor T lymphocytes by intragastric administration of soluble proteins. Journal of Immunology 121:2429-2434, 1978

83. Miller A, Lider O, Weiner HL: Antigen-driven bystander suppression after oral administration of antigen. J.Exp.Med. 174:791-798, 1991

84. Barone KS, Jain SL, Michael JG: Effect of *in vivo* depletion of CD4+ and CD8+ cells on the induction and maintenance of oral tolerance. Cellular Immunology 163:19-29, 1995

85. Chen Y, Kuchroo VK, Inobe J-I, et al: Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237-1240, 1995

86. Schwartz RH: A cell culture model for T lymphocyte clonal anergy. Science 248:1349-1356, 1990

87. Jenkins MK, Schwartz RH: Antigen presentation by chemically modified splenocytes induces antigen-specific unresponsiveness in vitro and in vivo. J.Exp.Med. 165:302-319, 1987

88. Quill H, Schwartz RH: Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. Journal of Immunology 138:3704-3712, 1987

89. Ramsdell F, Fowlkes BJ: Clonal deletion versus clonal anergy: The role of the thymus in inducing self tolerance. Science 248:1342-1348, 1990

90. Burstein HJ, Shea CM, Abbas AK: Aqueous antigens induce in vivo tolerance selectively in IL-2 and IFN-γ producing (Th1) cells. Journal of Immunology 148:3687-3691, 1992

91. Jenkins MK, Burrell E, Ashwell JD: Antigen presentation by resting B cells. Effectiveness at inducing T cell proliferation is determined by costimulatory signals, not T cell receptor occupancy. Journal of Immunology 144:1585-1590, 1990

92. Li Y, Yio XY, Mayer L: Human intestinal epithelial cell-induced CD8+ T cell activation is mediated through CD8 and the activation of CD8-associated p56lck. J.Exp.Med. 182:1079-1088, 1995

93. Harper HM, Cochrane L, Williams NA: The role of small intestinal antigen-presenting cells in the induction of T-cell reactivity to soluble protein antigens: association between aberrant presentation in the lamina propria and oral tolerance. Immunology 89:449-456, 1996

94. Blackman M, Kappler J, Marrack P: The role of the T cell receptor in positive and negative selection of developing T cells. Science 248:1335-1341, 1990

95. Sudo N, Sawamura S-A, Tanaka K, et al: The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. Journal of Immunology 159:1739-1745, 1997

96. Ferguson RM, Simmons RL: Differential cyclophosphamide sensitivity of suppressor and cytotoxic cell precursors. Transplantation 25:36-38, 1978

97. Rollinghoff M, Starzinski-Powitz A, Pfizenmaier K, et al: Cyclophosphamide-sensitive T lymphocytes suppress the in vivo generation of antigen-specific cytotoxic T lymphocytes. J.Exp.Med. 145:455-455, 1977

98. Gill HK, Liew FY: Regulation of delayed type hypersensitivity III. Effect of cyclophosphamide on the suppressor cells for delayed type hypersensitivity to sheep erythrocytes in mice. Eur.J.Immunol. 8:172-176, 1978

99. Mowat AM, Strobel S, Drummond HE, et al: Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology 45:105-113, 1982

100. Mattingly JA, Waksman BH: Immunological suppression after oral administration of antigen I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. Journal of Immunology 121:1879-1883, 1978

101. Andre C, Heremans JF, Vaerman JP, et al: A mechanism for the induction of immunological tolerance by antigen feeding: antigen-antibody complexes. J.Exp.Med. 142:1509-1519, 1975

102. Strobel S, Mowat AM, Drummond HE, et al: Immunological responses to fed protein antigens in mice II. Oral tolerance for CMI is due to activation of cyclophosphamide sensitive cells by gut-processed antigen. Immunology 49:451-456, 1983

103. Bruce MG, Ferguson A: The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. Immunology 59:295-300, 1986

104. Miller A, Al-Sabbagh A, Santos LMB, et al: Epitopes of myelin basic protein that trigger TGF-b release after oral tolerisation are distinct from encephalalitogenic epitopes and mediate bystander suppression. Journal of Immunology 151:7307-7315, 1993

105. Lunden BS, Dahlgren UIH, Hanson LA, et al: Oral tolerance leads to active suppression and bystander tolerance in adult rats while anergy dominates in young rats. Scand.J.Immunol. 43:56-63, 1996

106. Carvalho CR, Verdolin BA, De Souza AV, et al: Indirect effects of oral tolerance in mice. Scand.J.Immunol. 39:533-538, 1994

107. Thornton AM, Shevach EM: Suppressor effector functio of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. Journal of Immunology 164:183-190, 2000

108. Thornton AM, Shevach EM: CD4+CD25+ immunoregulatory T cells suppress polyclonalT cell activation *in vitro* by inhibiting interleukin 2 production. J.Exp.Med. 188:287-296, 1998

109. Mayer L, Shlien R: Evidence for the function of Ia molecules on gut epithelial cells in man. J.Exp.Med. 166:1471-1483, 1987

110. Lider O, Santos LMB, Lee CSY, et al: Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein II. Suppression of disease and *in vitro* immune responses is mediated by antigen specific CD8+ T lymphocytes. Journal of Immunology 142:748-752, 1989

111. Ke Y, Kapp JA: Oral antigen inhibits priming of CD8+ CTL, CD4+ T cells, and antibody responses while activating CD8+ suppressor T cells. Journal of Immunology 156:916-921, 1996

112. Chen Y, Inobe J-I, Weiner HL: Induction of oral tolerance to myelin basic protein in CD8- depleted mice: Both CD4+ and CD8+ cells mediate active suppression. Journal of Immunology 155:910-916, 1995

113. Vistica BP, Chanaud NP, Felix N, et al: CD8 T-cells are not essential for the induction of "low-dose" oral tolerance. Clin.Immunol.Immunopathol. 78:196-202, 1996

114. Grdic D, Hornquist E, Kjerrulf M, et al: Lack of local suppression in orally tolerant CD8-deficient mice reveals a critical regulatory role of CD8+ T cells in the normal gut mucosa. Journal of Immunology 160:754-762, 1998

115. Karpus WJ, Swanborg RH: CD4+ suppressor cells inhibit the function of effector cells of experimental autoimmune encephalitis through a mechanism involving transforming growth factor-β. Journal of Immunology 146:1163-1168, 1991

116. Hirahara K, Hisatsune T, Nishijima K-I, et al: CD4+ T cells anergized by high dose feeding establish oral tolerance to antibody responses when transferred in SCID and nude mice. Journal of Immunology 154:6238-6245, 1995

117. Desvignes C, Bour H, Nicolas J-F, et al: Lack of oral tolerance but oral priming for contact sensitivity to dinitrofluorobenzene in major histocompatability complex class II deficient mice and in CD4+ T cell-deplete mice. Eur.J.Immunol. 26:1756-1761, 1996

118. Mengel J, Cardillo F, Aroeira LS, et al: Anti-γδ T cell antibody blocks the induction and maintenance of oral tolerance to ovalbumin in mice. Immunol.Lett. 48:97-102, 1995

119. Ke Y, Pearce K, Lake JP, et al: γδ T lymphocytes regulate induction and maintenance of oral tolerance. Journal of Immunology 158:3610-3618, 1997

120. Wildner G, Hunig T, Thurau SR: Orally induced, peptide-specific γ/δ TCR+ cells suppress experimental autoimmune uveitis. Eur.J.Immunol. 26:2140-2148, 1996

121. Fujihashi K, McGhee JR, Kweon M-A, et al: γδ T cell-deficient mice have impaired mucosal immunoglobulin A responses. J.Exp.Med. 183:1929-1935, 1996

122. Hancock WW, Polanski M, Zhang J, et al: Suppression of insulitis in non-obese diabetic (NOD) mice by oral insulin administration is associated with selective expression of interleukin-4 and -10, transforming growth factor-β and prostaglandin-E. American Journal of Pathology 147:1193-1199, 1995

123. Fishman-Lobell J, Friedman A, Weiner HL: Different patterns of cytokine gene expression *in vivo* in orally tolerant mice. Eur.J.Immunol. 24:2720-2724, 1994

124. Yoshino S: Treatment with an anti-IL-4 monoclonal antibody blocks suppression of collagen-induced arthritis in mice by oral administration of type II collagen. Journal of Immunology 160:3067-3071, 1998

125. Inobe J-I, Slavin AJ, Komagata Y, et al: IL-4 is a differentiation factor for transforming growth factor-β secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. Eur.J.Immunol. 28:2780-2790, 1998

126. Claessen AME, Von Blomberg BME, De Groot J, et al: Reversal of mucosal tolerance by subcutaneous administration of interleukin-12 at the site of attempted sensitisation. Immunology 88:363-367, 1996

127. Marth T, Strober W, Kelsall BL: High dose oral tolerance in ovalbumin TCR-transgenic mice. Systemic neutralisation of IL-12 augments TGF-B secretion and T cell apoptosis. Journal of Immunology 157:2348-2357, 1996

128. Wolvers DAE, Van der Cammen JF, Kraal G: Mucosal tolerance is associated with, but independent of, up regulation Th2 responses. Immunology 3331997

129. Shi HN, Grusby MJ, Nagler-Anderson C: Orally induced peripheral nonresponsiveness is maintained in the absence of functional Th1 or Th2 cells. Journal of Immunology 162:5143-5148, 1999

130. Garside P, Steel M, Worthey EA, et al: T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. Journal of Immunology 154:5649-5655, 1995

131. Kehrl JH, Wakefield LM, Roberts AB, et al: Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. J.Exp.Med. 163:1037-1050, 1986

132. Kehrl JH, Roberts AB, Wakefield LM, et al: Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. Journal of Immunology 137:3855-3860, 1986

133. Chen Y, Inobe J-I, Marks R, et al: Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 376:177-180, 1995

134. Friedman A, Weiner HL: Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc.Natl Acad.Sci.USA 91:6688-6692, 1994

135. Miller A, Lider O, Roberts AB, et al: Suppressor T cells generated by oral tolerization to myelin basis protein suppress both the *in vitro* and *in vivo* immune responses by the release of transforming growth factor β after antigen specific triggering. Proc.Natl Acad.Sci.USA 89:421-425, 1992

136. Haneda K, Sano K, Tamura S, et al: TGF-β induced oral tolerance ameloriates experimental tracheal eosinophilia. Journal of Immunology 159:4484-4490, 1997

137. Fukaura H, Kent SC, Pietrusewicz MJ, et al: Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-B1 secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. Journal of Clinical Investigation 98:70-77, 1996

138. Barone KS, Tolarova DD, Ormsby I, et al: Induction of oral tolerance in TGF-β1 null mice. Journal of Immunology 161:154-160, 1998

139. Groux H, O'Garra A, Bigler M, et al: A CD4+ T-cell subset that inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737-742, 1997

140. Mosmann TR, Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today 17:138-147, 1996

141. Whitacre CC, Gienapp IE, Orosz CG, et al: Oral tolerance in experimental autoimmune encephalomyelitis III. evidence for clonal anergy. Journal of Immunology 147:2155-2163, 1991

142. Javed NH, Gienapp IE, Cox KL, et al: Exquisite peptide specificity of oral tolerance in experimental autoimmune encephalomyelitis. Journal of Immunology 155:1599-1605, 1995

143. Hanson DG, Miller SD: Inhibition of specific immune responses by feeding protein antigens. V. Induction of the tolerant state in the absence of specific suppressor T cells. Journal of Immunology 128:2378-2380, 1982

144. Melamed D, Friedman A: Direct evidence for clonal anergy in T lymphocytes tolerized by oral administration of ovalbumin. Eur.J.Immunol. 23:935-942, 1993

145. Inada S, Yoshino S, Haque MA, et al: Clonal anergy is a potent mechanism of oral tolerance in the suppression of acute antigen-induced arthritis in rats by the oral administration of the inducing antigen. Cellular Immunology 175:67-75, 1997

146. Van Houten N, Blake SF: Direct measurement of anergy of antigen-specific T cells following oral tolerance induction. Journal of Immunology 157:1337-1341, 1996

147. Sun J, Dirden-Kramer B, Ito K, et al: Antigen-specific T cell activation and proliferation during oral tolerance induction. Journal of Immunology 162:5868-5875, 1999

148. Melamed D, Friedman A: *In vivo* tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. Eur.J.Immunol. 24:1974-1981, 1994

149. Williams ME, Shea CM, Lichtman AH, et al: Antigen receptor mediated anergy in resting T lymphocytes and T cell clones - correlation with lymphokine secretion patterns. Journal of Immunology 149:1921-1926, 1992

150. Chen Y, Inobe J-I, Kuchroo VK, et al: Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependant induction of regulatory cells. Proc.Natl Acad.Sci.USA 93:388-391, 1996

151. Williamson E, O'Malley JM, Viney JL: Visualising the T-cell response elicitied by oral administration of soluble protein antigen. Immunology 97:565-572, 1999

152. Chen YH, Weiner HL: Dose-dependent activation and deletion of antigen-specific T cells following oral tolerance. Annals of the New York Academy of Sciences 778:111-121, 1996

153. Gregerson DS, Obritsch WF, Donoso LA: Oral tolerance in experimental autoimmune uveoretinitis. Distinct mechanisms of resistance are induced by low dose vs high dose feeding protocols. Journal of Immunology 151:5751-5761, 1993

154. Melamed D, Fishman-Lobell J, Uni Z, et al: Peripheral tolerance of Th2 lymphocytes induced by continuous feeding of ovalbumin. International Immunology 8:717-724, 1996

155. Evavold BD, Allen PM: Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. Science 252:1308-1310, 1991

156. Lombardi G, Sidhu S, Batchelor R, et al: Anergic T cells as suppressor cells *in vitro*. Science 264:1587-1589, 1994

157. Chai J-C, Bartok I, Chandler P, et al: Anergic T cells act as suppressor cells *in vitro* and *in vivo*. Eur.J.Immunol. 29:686-692, 1999

158. Matsui M, Hafler DA, Weiner HL: Pilot study of oral tolerance to keyhole limpet haemocyanin in humans. Down regulation of KLH-reactive precursor-cell frequency. Annals of the New York Academy of Sciences 778:398-404, 1996

159. Lowney ED: Immunologic unresponsiveness to a contact sensitizer in man. Journal of Investigative Dermatology 51:411-417, 1968

160. Lowney ED: Suppression of contact sensitization in man by prior feeding of antigen. Journal of Investigative Dermatology 61:90-93, 1973

161. Scott H, Rognum TO, Midtvedt T, et al: Age-related changes of human serum antibodies to dietary and colonic bacterial antigens measured by an enzyme-linked immunosorbent assay. Acta Pathologica Microbiologica et Immunologica Scandinavia 93:65-70, 1985

162. Marx J: Testing of autoimmune therapy begins. Science 252:27-28, 1991

163. Zhang ZJ, Davidson L, Eisenbarth G, et al: Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc.Natl Acad.Sci.USA 88:10252-10256, 1991

164. Sayegh MH, Zhang ZJ, Hancock WW, et al: Down-regulation of the immune response to histocompatibility antigens and prevention of sensitization by skin allographs by orally administered alloantigen. Transplantation 53:163-166, 1992

165. He Y-G, Mellon J, Niederkorn JY: The effect of oral immunization on corneal allograft survival. Transplantation 61:920-926, 1996

166. Trentham DE, Dynesius-Trentham RA, Orav EJ, et al: Effects of oral administration of type II collagen on rheumatoid arthritis. Science 261:1727-1730, 1993

167. Weiner HL, Mackin GA, Matsui M, et al: Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. Science 259:1321-1324, 1993

168. Sieper J, Kary S, Sorensen H, et al: Oral type II collagen treatment in early rheumatoid arthritis. A double-blind, placebo controlled, randomized trial. Arthritis and Rheumatism 39:41-51, 1996

169. McKown KM, Carbone LD, Kaplan SB, et al: Lack of efficacy of oral bovine type II collagen added to existing therapy in rheumatoid arthritis. Arthritis and Rheumatism 42:1204-1208, 1999

170. Hauselmann HJ, Caravatti M, Seifert B, et al: Can collagen type II sustain a methotrexate-induced therapeutic effect in patients with long-standing rheumatoid arthritis? A double blind, randomised trial. British Journal of Rheumatolgy 37:1110-1117, 1998

171. Barnett ML, Kremer JM, St Clair W, et al: Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo controlled trial. Arthritis and Rheumatism 41:290-297, 1998

172. Nussenblatt RB, Gery I, Weiner HL, et al: Treatment of uveitis by oral administration of retinal antigens: results of a phase I/II randomised masked trial. American Journal of Ophthalmology 123:583-592, 1997

173. Zhang J, Markovic-Plese S, Lacet B, et al: Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J.Exp.Med. 179:973-985, 1994

174. Lehmann PV, Forsthuber T, Miller A, et al: Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 358:155-157, 1992

175. Tisch R, Yang X-D, Singer SM, et al: Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 366:72-75, 1993

176. Devery JM, Geczy CL, Skerritt JH, et al: Macrophage procoagulant activity as an assay of cellular hypersensitivity to gluten peptides in coeliac disease. Clinical & Experimental Immunology 82:333-337, 1990

177. Devery JM, Bender V, Penttila I, et al: Identification of reactive synthetic gliaden peptides specific for coeliac disease. International Archives of Allergy & Applied Immunology 95:356-362, 1991

178. Maki M: Humoral immune system, in Howdle PD. (ed): Coeliac Disease. London, Bailliere Tindall, 1995, pp 231-249

179. Dieterich D, Ehnis T, Bauer M, et al: Identification of tissue transglutaminase as the autoantigen of coeliac disease. Nature Medicine 3:797-801, 1997

180. Parronchi P, Romagnani P, Annunziato F, et al: Type 2 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. American Journal of Pathology 150:823-832, 1997

181. Whittingham S, Feery B, Mackay IR: Use of tetanus toxoid for testing cell-mediated immunity. Australian and New Zealand Journal of Medicine 12:511-514, 1982

182. Jarvis JN, Zhao L, Moore HT, et al: Regulation of cytokine mRNA expression in activated lymphocytes by human choriocarcinoma JAR cells. Cellular Immunology 168:251-257, 1996

183. Boulton-Jones JM, Vick R, Cameron JS, et al: Immune responses in uraemia. Clinical Nephrology 1:351-360, 1973

184. World Health Organisation: Immunodeficiency, in AnonymousTechnical Report Series. Geneva, WHO, 1978,

185. Dawson ME: Endotoxin limits. LAL Update 13:1-3, 1995

186. Sigma-Aldrich. Sigma Aldrich Datasheet. 1998.

187. Kovacsovics-Bankowski M, Clark K, Benacerraf B, et al: Efficient major histocompatibiltity complex class I presentation of exogenous antigen upon phagocytosis by macrophages. Proc.Natl Acad.Sci.USA 90:4942-4946, 1993

188. Ke Y, Li Y, Kapp JA: Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. Eur.J.Immunol. 25:549-553, 1995

189. Kraft SC, Rothberg RM, Knauer CM, et al: Gastric acid output and circulating anti-bovine serum albumin in adults. Clinical & Experimental Immunology 2:321-330, 1967

190. Go C, Lancki DW, Fitch FW, et al: Anergized T cell clones retain their cytolytic ability. Journal of Immunology 150:367-376, 1993

191. Picker LJ, Treer JR, Ferguson-Darnell B, et al: Control of lymphocyte recirculation in man I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition. Journal of Immunology 150:1105-1121, 1993

192. Malavasi F, Funaro A, Roggero S, et al: Human CD38: a glycoprotein in search of a function. Immunology Today 95:95-97, 1994

193. Wallace DL, Beverley PCL: Phenotypic changes associated with activation of CD45RA+ and CD45RO+ cells. Immunology 69:460-467, 1990

194. Hanson DG, Vaz NM, Rawlings LA, et al: Inhibition of specific immune responses by feeding protein antigens II. Effects of prior passive and active immunisation. Journal of Immunology 122:2261-2266, 1979